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THE BIOCHEMICAL JOURNAL

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Papers submitted should be written concisely. Special attention is directed to *Policy of the Journal and Instructions to Authors*, *Biochem. J.* (1966), **98**, 1 (obtainable from the Secretary to the Editorial Board, The Biochemical Journal, 20 Park Crescent, London, W. 1, price 2*s.* 6*d.* post free).

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p.t.o.

Abbreviations. New abbreviations should be coined only for unwieldy names, and should not be used at all unless those names occur frequently. All abbreviations for names of compounds except those listed below must be defined together in a footnote.

In the title and synopsis, abbreviations should be avoided. Abbreviations for units (e.g. min., cm., g.) should be used throughout. Chemical symbols as abbreviations for elements, groups or compounds are used only in the Experimental section, and in legends to Tables and Figures, unless their use elsewhere is justified by special circumstances.

The following may be used without definition:

ADP, CDP, GDP, IDP, UDP	5′-Pyrophosphates of adenosine, cytidine, guanosine, inosine, uridine
AMP etc.	Adenosine 5′-phosphate etc.
ATP etc.	Adenosine 5′-triphosphate etc.
CM-cellulose	Carboxymethylcellulose
CoA and acyl-CoA	Coenzyme A and its acyl derivatives
DEAE-cellulose	Diethylaminoethylcellulose
DNA	Deoxyribonucleic acid
DNP-	2,4-Dinitrophenyl-
EDTA	Ethylenediaminetetra-acetate
FAD	Flavine–adenine dinucleotide
FMN	Flavine mononucleotide
GSH, GSSG	Glutathione, reduced and oxidized
NAD	Nicotinamide–adenine dinucleotide
NADP	Nicotinamide–adenine dinucleotide phosphate
NMN	Nicotinamide mononucleotide
RNA	Ribonucleic acid
P_i, PP	Orthophosphate, pyrophosphate
tris	2-Amino-2-hydroxymethylpropane-1,3-diol

Abbreviations for amino acids and sugars (see *Instructions to Authors*) are for use only in representing polymers or sequences and in Tables and Figures, and need not be defined.

Abstracts. For the convenience of authors, two extra copies of the synopsis can be submitted as abstracts. Authors are asked to submit with their typescript two such abstracts suitable for inclusion in *International Abstracts of Biological Sciences* and *Chemical Abstracts*. These abstracts will not appear in the *Biochemical Journal* but will be edited before being passed for publication. An abstract should be typed in double spacing on a separate quarto sheet in the following form: title, name(s) of author(s); *Biochem. J.* (space for year, volume and page reference); address (for reprint applications); synopsis.

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CORRECTIONS

The localization of adenosine triphosphatases in morphologically characterized subcellular fractions of guinea-pig brain

By R. Jeanette A. Hosie

Volume 96 (1965)

p. 405, column 1, para. 5 up, line 2: *for* P_2BW_s *read* P_2BW

p. 407, Table 3, column 1, lines 2 and 3: *for* P_2W_s *read* P_2W_p
for P_2W_p *read* P_2W_s

The fluorimetric determination of oxalic acid in blood and other biological materials

By P. M. Zarembski and A. Hodgkinson

Volume 96 (1965)

p. 717, column 2, last line: *for* 0·8 ml. *read* 0·08 ml.

The chemical structure of pemptoporphyrin

By S. Sano, T. Shingu, J. M. French and Elizabeth Thonger

Volume 97 (1965) pp. 250–256

The chemical name for pemptoporphyrin should be:

either 1,3,5,8-tetramethyl-2(4)-vinylporphin-6,7-dipropionic acid
or 2(4)-vinyldeuteroporphyrin IX

A search for *O*-polypeptidyl-ribonucleic acids in rabbit-reticulocyte ribosomes by electrophoresis in phenol–acetic acid–water systems

By Inger Brattsten, R. L. M. Synge and W. B. Watt

Volume 97 (1965)

p. 687, Scheme 2, equation (4): insert parenthesis immediately after arrow

Selective degradation of thymidine and thymine deoxynucleotides

By K. Burton and W. T. Riley

Volume 98 (1966)

p. 74, Table 3, heading for columns 5–8:

for Distribution of cytosine among products detected (%)
read Distribution of C_nP_{n+1} products (moles/100 moles)

p. 75, column 1, line 8: *for* 1·2:2 *read* 1·2:1

PROFESSOR ALBERT SZENT-GYÖRGYI

(*Facing p.* 641)

Biochem. J. (1966) **98**, 641

Growth and Organization

By ALBERT SZENT-GYÖRGYI*

THE FIFTH HOPKINS MEMORIAL LECTURE

Delivered on 1 *April* 1965 *in University College London, Gower Street, London, W.C.* 1

In the spring of 1926 I had to resign the very modest job I held at the University of Groningen. I had no means, no reputation, no knowledge, only a few insignificant papers, a family to support, and a burning desire to become a scientist. Losing my post meant the end of my scientific career. Since life and work meant the same to me I planned to give up both, simultaneously. So, I sent my wife home with our child to her parents and prepared for the end. As a farewell party, I went to the International Physiological Congress at Stockholm, presided over by Sir Frederick Gowland Hopkins. In his presidential address, to my surprise, he mentioned my name several times. So after his lecture I picked up my courage and went up to him. There I was, facing this wonderful little man, always completely forgetful of himself and his greatness, addressing me as his equal: 'Why don't you come to Cambridge? Ask for a Rockefeller Fellowship—I will see to it that you get it.' So he did, making England my spiritual homeland, where I learned what science is, where I was exposed, for several years, to that wonderful inspiration which radiated from him. His almost uncanny insight, prophetic vision, utter modesty and humility added up to a greatness which would have been depressing had it not been blended with warmth and charming human shortcomings which made him our beloved 'Hoppy'. He is still a mystery to me and I want to keep him that way, deep down in my heart.

In his presidential address to the British Association in 1913 you find the following sentence: 'Underlying the extreme complexity we may discover a simplicity which escapes us.' I feel strongly that this basic simplicity, the 'simplicité caché' of Poincaré, the unity contained in diversity, is much deeper-going than we envisage today. It has become, lately, of prime import to my Laboratory because we have obtained, from various tissues, extracts which retarded or promoted growth. The hypothetical substance responsible for retardation we called 'retine', and the promoting one we called 'promine'. We found such actions widely distributed in Nature, and the similarity in behaviour suggested that these activities were all due to identical or closely related simple compounds of low molecular weight. Could it then be that these were actually responsible for regulating cell division in the adult, and that proliferation should be regulated by the same set of simple substances throughout a living world which is characterized by its dazzling variety in shape, colour and function? This seems possible only if there is a deep-going unity in this world which still 'escapes us'.

In unicellular organisms growth regulation is relatively simple. It is one of the characteristics of life that it tends to spread and proliferate. Accordingly, in single cells multiplication is unlimited, being checked under favourable conditions only by the quantity of food and energy available. But when cells got together to form multicellular organisms, became members of an ordered society, they had to give up their unlimited proliferation, subject it to strict regulation. They also had to give up motility, and had to develop a new kind of surface which linked them to their neighbours with whom they had to live in the most intimate relations.

However, these newly acquired qualities could not be ingrained very deeply in an irreversible fashion, because, under certain conditions, the cells may be called upon to resume, at short notice, the unicellular way of living with its motility and uninhibited proliferation, which means reverting to the earlier evolutionary level. This is the case in regeneration, in the healing of a wound. In your quiescent epidermis, for instance, the cells are tough and are strongly bound together, which lends to your skin the toughness which you need for your protection. With some tanning, your skin would even make good boots. But if you cut yourself the whole situation changes. On the side of the cut the cells become free, resume motility, creep away into the wound with amoeboid motion, and proliferate. But this they do only till the wound is filled, is healed, and cell is surrounded by cell. Then everything stops and the initial multicellular state is restored. The cells have retained the ability to shift back and forth between the two states which correspond to their two evolutionary levels. The

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transition from one to the other is so prompt that it gives the impression of a veritable switch mechanism.

Conditions in a tissue culture are, in many ways, similar to the conditions in a healing wound, and all these relations were nicely spread out before us, so to say, by the tissue cultures prepared by Abercrombie and his associates. In cultures, too, cells make themselves free on the surface of the explant, resume motility, creep away and divide. They, however, do so only till the cells of neighbouring explants touch one another, which corresponds to the healing of the wound. When this stage is reached, everything comes to rest. This is what Abercrombie called 'contact inhibition'.

Looking upon the healing of a wound and regeneration as an alternation between two evolutionary states brings into the picture a 'unity in diversity', an underlying simplicity which may make the idea acceptable that in spite of the apparent diversity the same simple substances may be instrumental in the regulation.

All these relations assume a tragic importance through the fact that under the influence of various factors cells may lose irreversibly their latest, and thus least deep-seated, evolutionary accomplishment—their regulation of growth, turning into cancer. Looking upon cancer as an evolutionary state would allow us to view it scientifically.

All this makes it most urgent to get an insight into these relations and the question is how this problem can be approached. I am unable to see how it could be approached at all. It is too complex, subtle and full of pitfalls. If we cannot approach a problem, all we can do is to wait till the problem approaches us, and I would like to tell the story, briefly, how I and my associates, Dr Andrew Hegyeli, Dr Laszlo Együd, Dr Ruth Johnsson and Miss McLaughlin, were approached by it.

The main interest of my Laboratory was, for a long time, muscle, and clinical experience indicates that the thymus gland is, somehow, involved in its function. So I set out hunting for a thymus hormone. I was hunting for something of which I did not know, what it was, what it did, and whether it existed at all. As test animals I chose myotonic goats, but soon this line had to be given up for the simple reason that I work in a marine biological laboratory and the smell of goats clearly identifies them as non-marine organisms. At that time I speculated about electronic states and believed that my hypothetical thymus substances had to be coloured, i.e. capable of easy electronic excitation. This led me to the isolation of a beautiful yellow substance which, on analysis, turned out to be an uninteresting derivative of ascorbic acid. This disappointment made me shift to fluorescence and soon I discovered a wonderful fluorescent substance present in my extracts in trace amounts. My only brilliant chemical work, of which I am conscious, was the isolation of this substance in crystals. The constitution analysis showed it to be a substance which I extracted from my rubber tubing. Disgusted with these theories I argued thus: growth is the integral of all functions and so any biological activity in my extracts should declare itself by modifying growth. In order to get fast results I had to use a fast-growing tissue, and so I chose cancer. The test for activity was simple. A number of Swiss albino mice were injected, behind the scapula, with a definite number of Krebs II ascites-cancer cells. Four days later a visible and palpable tumour appeared, and daily injections of extracts were begun. Seven days later the animals were killed, the tumours excised, inspected, weighed and studied microscopically, and the weights tabulated. Retine makes the cells die, promine makes the tumour grow faster. A 50% retardation or promotion of growth was called a 'unit' of activity. We also tested other solid tumours, e.g. sarcoma 180 and the spontaneous tumours of C3H mice. All reacted similarly. The percentages observed seemed to be a function of the logarithm of the quantity of retine. The results thus obtained could also be checked by injecting cancer cells into the peritoneal cavity and observing the extent of ascites and time of survival. We also made ample use of the tissue cultures prepared by Dr Johnsson (KB and HeLa cells). Retine inhibits growth or eliminates it altogether, whereas promine causes a slight increase in cell volume and a spreading of the cells.

These methods enabled us to express action numerically. The results were numeric but showed the most disconcerting inconsistency. Most of the extracts inhibited, but some promoted, and even one and the same extract could turn, on storage, from an inhibitor into a promoter. It took a great deal of the classical mixture of inspiration and perspiration till we could see that we were dealing with two substances with opposite activity. During the preparative work, also, the physical properties of these substances often changed completely and it dawned on us, but slowly, that in the animal both are loosely linked to a colloid from which they can dissociate, introducing a new variable. One variable can easily be managed, but two may create major difficulties. We had about half a dozen. So I will cut my story short and jump to the final results and then work my way backwards. I will speak first about retine, the inhibitor.

The physical properties of retine, while linked to its colloid, reflect the properties of its carrier. It is soluble in water, can be purified without loss by dialysis, can be precipitated by higher concentrations of ethanol or acetone, is carried down from crude extracts by ammonium sulphate. On

Sephadex G-25 it shows a slight retardation, on Sephadex G-100 a stronger one, suggesting a colloid of moderate size with a molecular weight of several thousand. When detached from this colloid the retine readily passes the dialysis membrane, shows a strong retardation on Sephadex, and can readily be shaken out from its moderately acid watery solution by chloroform or related solvents. Its behaviour, under various conditions, suggests a molecular weight of a few hundred. It distils over with acetic acid. It resists boiling, but if treated with strong acid or alkali at higher temperatures it changes properties. Acid treatment makes it very toxic, whereas treatment with alkali seems only to decrease its activity. Both treatments lower the boiling point and make the retine distil over even with chloroform, indicating a very low molecular weight. The infrared spectrum of our purest preparations suggests a ketone with hydroxyl groups and non-conjugated double bonds, but no nitrogen and no aromatic structure. All active fractions showed, hitherto, a positive Schiff reaction and the slow formation of a 2,4-dinitrophenylhydrazone. An absorption at about 272 mμ seems to accompany activity. At a strongly alkaline pH retine can be distilled from the crude extracts with steam. Retine has a high biological activity. The weight of a unit is of the order of micrograms. It seems to act equally well when given by mouth and parenterally. Both retine and promine, at higher purity, seem to be devoid of toxic side-effects.

Promine shares all known physical and chemical properties with retine, which makes the separation of the two very difficult. Effective separation can be achieved by distillation at a strongly alkaline pH, under which conditions the retine goes over more readily into the distillate. Separation can also be achieved by chromatography on paper. On Sephadex, promine tends to move more slowly than retine. The great similarity of the two substances indicates that they are closely related, or identical with only some minor difference in structure, which might make it possible for Nature to shift readily from inhibition to promotion, providing a sort of switch mechanism. In all tissues hitherto studied we found some promine accompanying retine and we believe that there is an equilibrium between the two which decides the biological activity of an extract. In all tissues studied we found retine in excess, the extracts having a retarding action, which corresponds to the quiescent state of tissue from which the extracts were obtained. Promine can completely compensate for the action of retine and vice versa. So the inhibitory action of an extract does not necessarily indicate the absolute quantity of retine or promine, but only the difference between the activity of the two substances. It will be important to bear this in mind when physiological and pathological relations are studied. We found, for instance, that our tumour grew faster in young than in old animals. To quote an experiment, the tumours grew, under identical conditions, to an average weight of 4823, 2735 and 542 mg. in mice 4 weeks, 4 months and 1 year old respectively. This difference between young and old could be eliminated by the administration of retine or promine. Though the relative concentrations of these substances may be identical at various ages, their absolute concentrations may be different, low in the old, high in the young, responsible for the greater incidence of cancer in the former and its rapid growth in the latter.

As a material for our preparations we used, at the outset, thymus glands. We also made extracts from big blood vessels, muscle and tendon (the three tissues which contract cancer least frequently) and found retine prevailing in all three. This convinced us that retine is not a thymus hormone but a general tissue constituent. In the thymus we found one-third retine unit/g., which is, in our experience, roughly the concentration which is needed in the animal to stop cancer growth, suggesting that it may actually be the retine which keeps tissues in the quiescent state.

This brings me to one of the difficulties of these studies. When attempting to isolate a hormone one can go to its source, the gland which produces it, where we usually find it in relatively high concentration. There is no such gland for retine, which seems to be produced locally by the various tissues in trace amounts, which have to be separated thus from the overwhelming mass of other tissue constituents, to which it adsorbs most readily. So when our friend Dr Charles Huggins suggested that we try urine as material we welcomed the idea. For theoretical reasons, we used, first, urine of little children, but shifted, soon, for practical reasons, to an older age group of 20–25, using the urine produced by the U.S. Army, which was made accessible to us by Abbott Laboratories in quantities of 5000 gallons/week. About 25–50 ml. contained 1 unit of retine. The greatest part of this we found linked to its colloidal carrier. The free retine present could be adsorbed to charcoal and eluted with chloroform, while the colloid-bound retine was carried down with the bulky precipitate which is formed when the urine is concentrated to one-tenth or one-fiftieth of its original volume. Boiling this precipitate with acid releases the retine, both from the precipitate and its colloidal carrier.

Folklore attributes a favourable action to mushrooms in cancer. This made us try this material, which we found rich in retine. Using *Agaricus campestris*, the edible mushroom, we found in the pressed juice 3 units/ml., accompanied by relatively little promine. This retine shares all the physical

and chemical properties with that prepared from animal tissues or urine. The host of accompanying toxic substances makes mushrooms an unfavourable material. So we dropped it after having convinced ourselves that retine may also be found in plants.

Sister Rosarii Schmeer, working at Woods Hole, heard about our results and noted that no malignant growth was yet reported in molluscs. So she set out to study clams for an anticancer activity, using chiefly quahog, *Mercenaria mercenaria*. She found a definite activity. We followed her trail and corroborated her results. We found, in the summer, in 1ml. of the homogenized content of the valves, about 3 units of retine. Two-thirds of this was in the clam's body, about equally distributed in the various organs. One-third was in the valve fluid. This retine shares all the chemical and physical properties with the retine found in vertebrates or mushrooms. It is linked *in vivo* to a colloid of similar dimensions. Also, the clam is relatively poor in promine.

All this shows that retine is widely spread in Nature. The remarkably high retine content of mushrooms and clams suggests that this substance may have a function other than the regulation of cell division. Maybe it is used as a defence against the attack of invading micro-organisms, to which both clams and mushrooms are exposed, the one living in mud, the other in decaying matter. This unequal distribution of retine makes a wide search in Nature for anticancer substances warranted.

To sum up: the research conducted in my Laboratory, during the past score of years, has brought to light two closely related substances, widely distributed in Nature, which substances seem to be instrumental in the regulation of cell division in the adult multicellular organism. The hope seems to be justified that more intimate knowledge of them will open up a new alley for attack on the subtle problems of cellular growth and intercellular relations, and the related problems of DNA replication and protein synthesis. A reliable micro-method for their estimation may help to shed light on fascinating problems of oncology. If I did not touch upon any of these, this is for a simple reason. It is not impossible that retine will be helpful in controlling malignant growth, but it can hardly be of any practical use while we are dependent for its production on natural sources. The human suffering caused by cancer entails the obligation to follow the narrow road which leads over isolation and constitution analysis to synthesis. Until this is achieved one has to shy away from all the fascinating functional problems. But the chemical outlines of these growth regulators begin to emerge and I do think that research on this line has passed its most difficult stages, and the story will be completed in the not too distant future.

When I accepted the invitation to this Lecture, more than a year ago, I hoped to be able to finish it with a happy end, pulling out of my pocket a bottle of the synthetic product. This was not to be. The problem is the most difficult one I have ever met. Going was rough and slow. Perhaps I could have found, in my past, some more finished work to talk about, but Hopkins never stopped to delight himself in past achievements. He was always future-oriented. So if I only took you into my workshop, and leave you with more questions asked than answered, at least I have not acted contrary to the spirit of my beloved teacher.

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The Cyst Wall of *Colpoda steinii*

A SUBSTANCE RICH IN GLUTAMIC ACID RESIDUES

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1. The cyst wall of *Colpoda steinii* has been isolated and its chemical nature examined. It had a nitrogen content 13·9 ± 0·2% (S.D.) and an ash 8·6 ± 1·6% (S.D.). After lipid and hot-acid extraction there was a variable residual phosphorus of 0·19–0·64%. The protein nature, indicated by infrared and ultraviolet absorption, was confirmed when 100 μg. of hydrolysed wall gave a ninhydrin colour equivalent to that given by 0·88–1·01 μmoles of glycine. Hexosamine, hexose, pentose, lipid and dipicolinic acid were absent. 2. Paper chromatography of hydrolysates, besides showing the presence of the usual protein amino acids and three unidentified ninhydrin-reacting spots, indicated the presence of large amounts of glutamic acid. Estimated by chromatography, the amount present was 52·9 ± 0·6 (S.D.) g./100g. of ash-free wall; manometric estimation of L-glutamic acid with L-glutamate 1-carboxy-lyase gave 46·5 ± 0·9 (S.D.) g./100g. 3. Free carboxyl groups were estimated by titration as 0·159 ± 0·011 (S.D.) mole/100g. and those present as amide as 0·154 ± 0·004 (S.D.) mole/100g., and the total was compared with the dicarboxylic acid content 0·360 ± 0·010 (S.D.) mole/100g. 4. After treatment with 98% formic acid 25–30% of the wall material could be extracted by 0·05 M-sodium carbonate solution (extract 1); after treatment of the residue with performic acid a further 62–63% based on the original weight could be extracted by 0·05 M-sodium carbonate (extract 2). 5. The average values found for the glutamic acid contents were 21·7g./100g. for extract 1 and 58·0g./100g. for extract 2. The cysteic acid content of whole oxidized wall was about 5·8g./100g. and of extract 2 also about 5·8g./100g. The glutamic acid and cysteic acid contents of the final residue were also investigated. 6. The significance of these extraction experiments in relation to the wall structure is discussed.

The various species of the free-living protozoan *Colpoda* may be stimulated by unfavourable changes in environment to produce resistant encysted forms. The most important of these changes is the shortage of food (Taylor & Strickland, 1938) and cells will become rounded, absorb their surface structures and begin the secretion of a thick protective wall within a few minutes of washing away the culture fluid. Many other Protozoa show a similar behaviour and the dramatic change in morphology and metabolism accompanying the encystment process may form a useful model in a study of cellular differentiation (Trager, 1963).

Older work on the nature of the cyst walls of free-living Protozoa (Stein, 1854; Fabré, 1885; Goodey, 1913) stressed the possible presence of carbohydrate materials, as did the observations of Sebestyén (1935) on *Diplopsalis acuta*. More recently cellulose has been identified as a component of the wall of *Acanthamoeba* sp. (Tomlinson & Jones, 1962) and chitin has been claimed to be present in the cyst membranes of *Pelomyxa illinoisensis* (Sachs, 1956).

This paper describes the growing in substantial quantities of the organism *Colpoda steinii* and the isolation and chemical investigation of wall preparations from the encysted form of this organism. Short accounts of some aspects of the work have already been published (Tibbs, 1964, 1965).

METHODS

Isolation of Colpoda steinii. *C. steinii* was isolated from dry pond grass which had been exposed to air as the result of drought. This grass was immersed in water in a Petri dish and left for 1–2hr., when excystment of *Colpoda* occurred, the larger forms being easily distinguishable under the dissecting microscope from the smaller *C. steinii* and *C. aspera* (Burt, 1940). One of the smaller forms was picked out with a Pasteur pipette and washed by transferring it through several drops of water in cavities on microscope slides. The organism was then placed in a drop of *Cloaca*

cloacae suspension and kept in a humid atmosphere at room temperature. After a period of rapid multiplication encystment took place on the third to fourth days. The cysts adhered to the glass surface and the supernatant was removed and replaced by a drop of hay infusion. Within less than 2 hr. the drop was rich in motile forms of *Colpoda*.

The organisms in this drop were used for species identification and for further subculturing and all the work described in this paper has been carried out on descendants of one cell isolated as described. A detailed morphological analysis has not been carried out but the organism averaged about 30 μ in length as indicated by Burt (1940) for *C. steinii*, and possessed the 'beard' of pseudomembranelles together with the long caudal cilia which are characteristic and unique for this species.

Culture of Colpoda steinii. The organism was grown in quantity by using *Cloaca cloacae* as food in the following manner. Ten litres of a solution of 0·2% Difco Bacto-Tryptone and 0·2% Difco yeast extract were distributed amongst a number of litre conical flasks so that each flask contained approximately 300 ml. of medium. After stoppering and autoclaving the flasks were inoculated with *Cloaca cloacae* (Challoner P11, N.C.T.C. 9238) and incubated for 24 hr. at 37°. The bacteria were then harvested by using a Sharples continuous centrifuge and washed by following the medium through the centrifuge with 2 l. of water. The concentrated suspension was filtered through coarse sintered glass to remove cotton-wool fibres etc. and made up to 2 l. with water. Suspensions so obtained contained 1·2–1·8 mg. dry wt. of bacteria/ml. and this concentration was optimum for subsequent growth of *C. steinii*. If the bacteria were suspended in a larger volume of water the subsequent yield of *Colpolda* was no greater and handling difficulties were increased. If the volume of the final suspension was made considerably less than this, growth was not rapid and final yields were reduced.

The suspension was now inoculated with a washed motile preparation of *C. steinii* obtained by decanting the supernatant fluid from an encysted culture and treating this with hay infusion. With cultures aged about 2 weeks excystment occurred in 1½–2 hr. at 25°. With older cultures or cultures which had been allowed to dry out excystment took longer.

The suspension was now spread on flat-bottomed Pyrex dishes; the depth of liquid in each dish was kept to a minimum (about 1 mm.), for growth in deeper layers was slow, encystment uncertain and yields were poor. The dishes were made level and kept at 25°. *C. steinii* multiplied rapidly and on the third to fourth days after inoculation most of the cells encysted fairly synchronously within an hour or so. The trays were kept a further 7 days to allow the encystment process to reach completion. The growth temperature was not critical.

Preparation of cyst wall. The contents of the dishes were now harvested, and centrifuged at about 1000*g* for 5 min. to spin down the cysts. These cysts were transferred to two 20 ml. tubes of a Mickle high-speed shaker and shaken with an equal volume of grade 4 Ballotini beads (approx. 1·0 mm. diam.) in a Mickle shaker for 15 min. The contents were then examined under phase contrast to make sure that cell rupture was complete. Occasionally the organic matter in the tubes was more concentrated and more viscous than usual and a longer period of shaking was necessary. Excessive shaking was inadvisable because powdered glass was produced and the wall itself became fragmented. The size of bead was important and other sizes from 0·1 to 2·0 mm. diam. were less effective.

The homogenate was filtered through grade 2 sintered glass (which retained the beads but allowed the wall material to pass through) and the filtrate spun at 500*g* for 5 min. The supernatant was discarded and the residue, consisting mainly of wall material, was washed several times by centrifugation until the supernatants were clear. The residue, which consisted of wall material to which a certain amount of particulate matter adhered, was suspended in a small volume of water and dispersed by a 10 sec. treatment with the probe of a 13 kcyc. ultrasonic oscillator (Ultrasonics Ltd., Otley, Yorks.). This short treatment dislodged much of the small particulate matter but did not further rupture the walls. The walls were again spun down at 500*g* for 5 min. and washed several times with water. The final suspension (Plate 1b) was freeze-dried for storage. Yields were 30–40 mg.

Maintenance of species. It was found that if sucrose solution (final concn. 0·25–0·75 M) was added to a cyst suspension in water the suspension could be frozen at −10° and the cells remained viable for many months. In the absence of sucrose, after a few days at −10° only a small proportion of cells was still viable. Repeated thawing and freezing of cultures stored with sucrose caused no obvious reduction in viability, which was tested by thawing, removing the supernatant by centrifugation and spreading the cysts with hay infusion in a Petri dish. With a preparation of viable cysts the dish, after an hour or two, would be teeming with motile organisms.

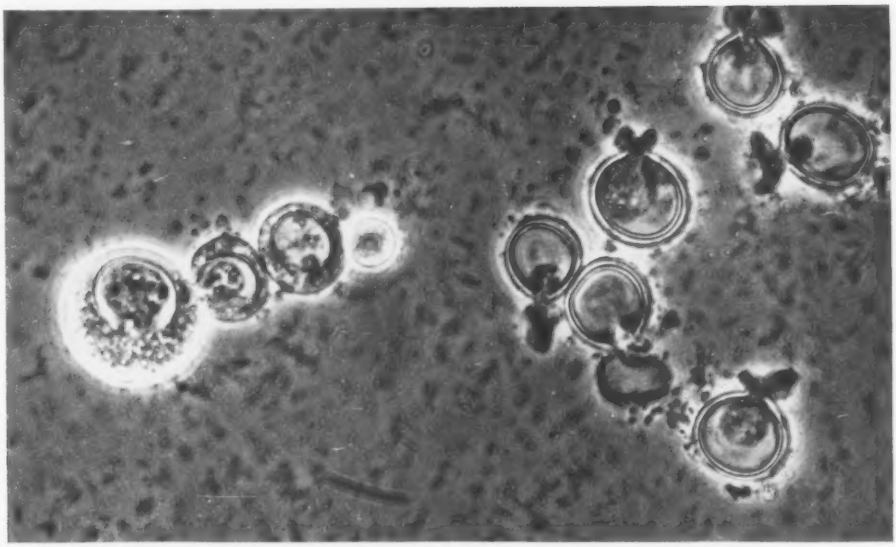
Analyses. Some ash contents and elementary analyses were carried out on the material dried for 2 hr. at 105° by Weiler and Strauss, Oxford. For analyses in the laboratory preliminary drying for 2 hr. at 105° was also employed. The ash content was taken as the residue obtained after heating at 550° for 2 hr. Total nitrogen was estimated by the micro-Kjeldahl method and phosphorus by the Allen (1940) procedure.

For the detection of hexosamine in the cyst wall an acid digest (16 hr. at 105° in 6 N-HCl) was dried *in vacuo* and an amount equivalent to 1 mg. applied as a spot to a strip of Whatman no. 1 filter paper. The chromatogram was developed in propan-2-ol–water (4:1, v/v), dried, and sprayed with the hexosamine (Elson–Morgan) reagent (Smith, 1960, p. 252). An amount of glucosamine equal to 2% by wt. of the cyst wall was added to a further sample of wall which was hydrolysed and chromatographed as just described. There was no difficulty in detecting glucosamine on this control chromatogram.

With glucose as standard, hexose was determined by the anthrone reaction (Trevelyan & Harrison, 1952). A 10 min. heating period with the reagent was used (Yemm & Willis, 1954) and during this the cyst wall was dissolved. With xylose as standard the reaction with orcinol (Albaum & Umbreit, 1947) was used to detect pentose. Lipid was estimated by measuring the loss in weight on extracting wall (10–20 mg.) at room temperature once with ethanol, twice with ethanol–chloroform (3:1, v/v), once with ethanol–diethyl ether (3:1, v/v) and once with diethyl ether.

The amount of material dissolving in two 10 min. extractions at room temperature with 0·5 N-perchloric acid was also determined. These extracts were made up to a known volume and the ultraviolet-absorption curve was plotted. Wall preparations were also tested for the spore compound

(a)



(b)

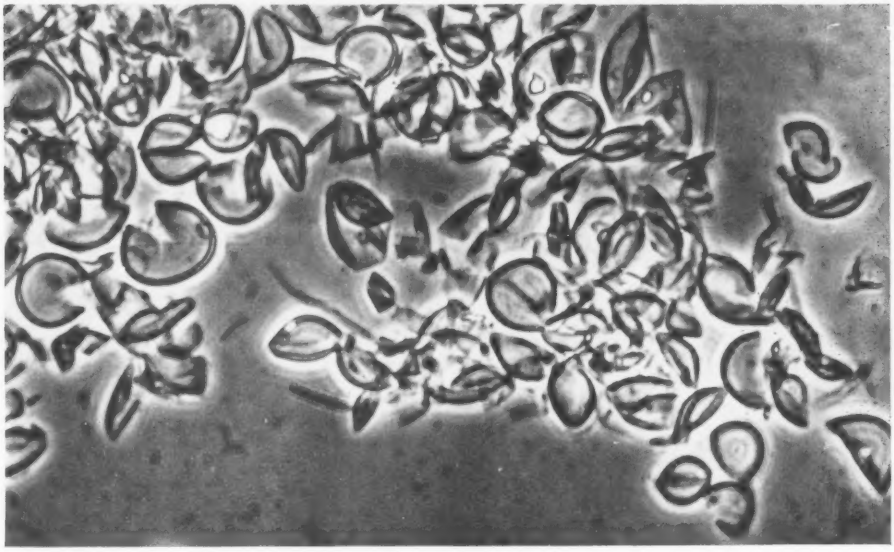


Plate 1. (a) Photomicrograph of partially ruptured *Colpoda steinii* cysts as seen in phase contrast. Cells were mounted in culture fluid and broken by applying pressure to the coverslip. On the left-hand side are three cells, one of which is highly swollen, in which the inner wall has been fractured but the cytoplasm has been retained by an external membrane. On the right-hand side are cells in which both the inner wall and external membrane have been fractured and from which the cytoplasm has been partially expelled. (×800) (b) Photomicrograph of *C. steinii* wall preparation in water as seen in phase contrast. The thick inner layer of the wall complex is the major component. (×800).

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(*Facing p.* 646)

dipicolinic acid by measuring the absorption of a hot aqueous extract (Powell, 1953). The ultraviolet absorption of the cyst wall itself at high and low pH values was recorded as follows. Wall (10 mg.) was extracted twice with 0·5 N-perchloric acid at 70° for 20 min. to remove nucleotide and nucleic acid absorbing material and then treated with 5 ml. of N-NaOH for 20 hr. at 37°. Undissolved material was removed by centrifugation at 15000*g* for 10 min. and discarded. This was dark-coloured and gritty, had a negligible nitrogen content and was assumed to consist of contaminating inorganic matter. Portions of the supernatant were taken for nitrogen estimation and for dilution with water or acid to give solutions, the pH values and ultraviolet-absorption curves of which were measured.

Paper chromatography was used to detect amino acids. Cyst walls and cyst-wall fractions were hydrolysed by boiling with 6 N-HCl for 24 hr. Humin was removed and after evaporating to dryness under partial vacuum hydrolysates were dissolved in water for chromatography. For two-dimensional work an amount of hydrolysate corresponding to 100–150 μg. of wall was used. Chromatography at 25° was by the ascending method and with a frame of the type described by Datta, Dent & Harris (1950). Solvents employed were butan-1-ol–acetic acid and phenol–NH_3. They were used in that order and made up as described by Smith (1960, p. 83). After drying chromatograms were sprayed with ninhydrin as described by Moffat & Lytle (1959). Desalting before chromatography was not necessary. Unidentified ninhydrin-reacting spots were further investigated by the pyridoxal transamination method of Kalyankar & Snell (1957).

Several amino acids (cysteic acid, aspartic acid, glutamic acid and glycine) were estimated by running one-dimensional descending chromatograms in phenol–NH_3. Loading of the chromatograms was carried out so that the acids to be estimated were present in amounts of 3–10 μg. and standards were run on the same sheet. After 24 hr. development at 25° phenol was removed and amino acids were estimated according to Kay, Harris & Entenmann (1956).

To determine the optical configuration of amino acids present in the cyst-wall hydrolysate the paper-chromatography method described by Boulanger & Osteux (1963) was used. The D-amino acid oxidase (EC 1.4.3.3) was a hog-kidney preparation from Sigma Chemical Co. and the catalase (EC 1.11.1.6) was from British Drug Houses Ltd. The same procedure was also used with an L-amino acid oxidase preparation (EC 1.4.3.2) (from snake venom, supplied by Sigma Chemical Co.). L-Glutamic acid was determined manometrically in hydrolysates with glutamate decarboxylase (EC 4.1.1.15) (Gale, 1963). The enzyme preparation was a crude acetone-dried *Escherichia coli* powder obtained from Sigma Chemical Co.

Ninhydrin reactions in solution were performed by the Moore & Stein (1948) procedure. Amide nitrogen was estimated by heating 5 mg. samples with 2 N-HCl in sealed tubes at 100° for 1–6 hr. The tubes were opened and HCl was removed *in vacuo* over P_2O_5 and NaOH. The NH_3 in each tube was estimated by the micro-diffusion technique (Conway, 1957) and the values were extrapolated to zero time to find the true amide nitrogen.

Each point on the titration curve was obtained as follows. A known amount of wall (approx. 10 mg.) was dispersed in 2 ml. of M-KCl and a known volume of 0·05 N-HCl or 0·05 N-KOH (both in M-KCl) added. Nitrogen was used to exclude CO_2 when the final pH was greater than 5. The tube containing the wall was sealed and incubated for 2 days at 30° and, after an accurate estimate of the volume of the liquid phase had been obtained by weighing, was centrifuged. A portion (1 ml.) of the supernatant was taken for titration of the free acid if the final pH was less than 4; between pH 4 and 10 the free acid was calculated from the pH of the supernatant. Reversibility was verified by adding that volume of titrant calculated to bring the system back to the original pH and 2 days at 30° was allowed for this equilibrium to be established.

Oxidation and extraction of wall preparations. Oxidation with performic acid was carried out as described by Thompson & O'Donnell (1959) for 16 hr. at 0°. The freeze-dried material was extracted for 48 hr. at 37° with 0·05 M-Na_2CO_3 (1 ml. to every 2 mg. of material). A drop of toluene was included in the suspension to reduce any bacterial contamination. The extract was removed by centrifuging at 50000*g* for 30 min. and the residue washed with water. Salt was removed from the combined extract and washings by dialysis and the extract and insoluble residue were freeze-dried. From two such experiments the diffusate was freeze-dried, refluxed with 6 N-HCl for 24 hr. and, after removal of the acid *in vacuo*, made up to a known volume and the reaction with ninhydrin investigated (Moore & Stein, 1948). Individual amino acids present in the hydrolysed diffusate were detected, after desalting in an electrolytic desalter, by the chromatographic procedure described above.

RESULTS

Disruption of cysts. The cyst wall of *Colpoda* consists of more than one layer. In intact cysts, but especially in partially disrupted suspensions, it is possible to see a smooth rigid wall within a thin membrane. The inner wall is fractured more easily than the outer membrane and a half-way stage in cyst rupture can frequently be seen, where the inner wall has been broken and allows cytoplasm to pass through, but the external membrane is still intact and retains the cytoplasm. When cysts are crushed under a coverslip both these layers may be broken and although the cytoplasm is released into the external medium it is not always immediately dispersed but often remains coherent with a smooth external surface. Presumably yet another membrane is involved. All these points may be seen in Plate 1(a). Plate 1(b) shows that the smooth rigid and comparatively thick component forms a major part of the final preparation, whereas the origin of some thinner and less rigid material that is also present is less certain.

The final preparation contained a small amount of particulate matter which was not removed by further washing with water, buffers or salt solutions.

General nature of cyst-wall material. The percentages of ash, carbon and hydrogen as determined on five different preparations by Weiler and Strauss were 8·6 ± 1·6 (S.D.), 42·1 ± 1·1 (S.D.) and 6·3 ± 0·2

(S.D.) respectively. The nitrogen content determined in this Laboratory on six batches of material was $13{\cdot}9 \pm 0{\cdot}2$ (S.D.). Hexosamine, if present, was less than 2% and determinations of hexose on four batches of wall gave values ranging from 1·8 to 3·0%. Similarly the pentose contents of four batches were between 0·9 and 1·5%. These small figures have doubtful significance and therefore hexosamine, hexose and pentose are essentially absent from the walls of *Colpoda* cysts. Lipid also appeared to be absent. Up to 6% loss in weight occurred during extraction of four batches of wall with cold acid. This extract absorbed in the ultraviolet with a maximum at 255mμ and presumably was nucleotide. Estimated as adenylic acid and by using a molar extinction coefficient $15{\cdot}0 \times 10^3$ (Bock, Ling, Morell & Lipton, 1956), the amount varied in four different batches but was always less than 0·7%. Hot-water extracts of wall (5mg.) showed some ultraviolet absorption with maxima again at 255mμ with no suggestion of a peak at about 270mμ; this excludes the presence of the bacterial spore compound dipicolinic acid (Powell, 1953).

Eleven batches of material contained 1·0–1·5% of phosphorus. Although much of this was extracted by treatment with lipid solvents and hot 0·5N-perchloric acid some was left behind. The amount remaining was variable and, based on the original weight of unextracted wall, was 0·2–0·6%.

Hydrolysates of three batches of cyst wall (100μg. quantities, corrected for ash) gave colours with ninhydrin equivalent to 0·96, 0·88 and 1·01μmoles of glycine. The ultraviolet-absorption curves measured at pH 0·6 and 12·7 on a batch of wall freed from nucleotide or nucleic acid absorbing material showed the profile of protein containing both tyrosine and tryptophan. The method of Beaven & Holiday (1952) could not be used to calculate with any confidence the amounts of these two amino acids, for when the two curves were corrected as suggested by these authors to take into account any non-specific absorption they crossed at 288mμ and not between 277 and 281mμ as would be expected for a normal protein. This, together with the observation that the curve in alkali showed what appeared to be an exceptionally high absorption at the longer wavelengths around 320mμ, suggests the presence of some other absorbing material and makes a calculation of the tyrosine and tryptophan content valueless. The infrared-absorption curves of two wall preparations made up as Nujol mulls were recorded with a Unicam SP.200 spectrophotometer and showed strong absorption at about 3300, 1660 and 1550cm.$^{-1}$. These absorptions are typical of a protein (Fraser, 1953).

Dr K. M. Rudall of the Astbury Department of Biophysics in the University of Leeds examined the X-ray powder diagram of a wall preparation and found that because of the presence of strong diffractions arising from inorganic material it was not possible to define with certainty any characteristic diffractions from the organic material. A wall preparation with a low ash content of 6·4% (not sulphated) was examined qualitatively by the Department of Spectrochemistry, Macaulay Institute for Soil Research, Aberdeen, and 25 elements were detected in the ash. Silicon and magnesium were possibly the main constituents but copper, lead, tin and zinc were also present in relatively large amounts. Such elements as calcium, potassium and sodium made only a minor contribution.

Ninhydrin-reacting substances present in hydrolysates. Chromatograms of cyst-wall hydrolysates showed considerable quantities of glutamic acid. Also present were aspartic acid, glycine, serine, threonine, alanine, tyrosine, lysine, arginine, histidine, valine, phenylalanine, leucine and proline. Three other unidentified ninhydrin-reacting spots were detected and the pyridoxal method of Kalyankar & Snell (1957) indicated that these spots were not due to α-amino acids.

The glutamic acid content of five preparations was $52{\cdot}9 \pm 0{\cdot}6$ (S.D.) g. from 100g. of ash-free wall. This value is high, for with the solvent system used (phenol–ammonia) glutamic acid would not have separated from one of the unknown ninhydrin-reacting spots. This spot was not strong and the error introduced is not serious.

A D-amino acid oxidase preparation when sprayed on to a chromatogram had no effect on a D-glutamic acid control spot, as judged by the lack of fluorescence on spraying with *o*-phenylenediamine in trichloroacetic acid but attacked D-alanine without difficulty. The enzyme had no detectable effect on a chromatogram of a cyst-wall hydrolysate. The L-amino acid oxidase preparation attacked both L-alanine and L-glutamic acid and after treating a chromatogram with this enzyme all major amino acid spots fluoresced when sprayed as before. It was concluded that much of the glutamic acid was in the L-configuration and that most likely all the other amino acids were completely in the L-form. Manometric estimation of the L-glutamic acid gave, for three batches of wall, 45·2, 46·6 and 47·7g. from 100g. of ash-free wall. The corresponding figures for glutamic acid, obtained by chromatography, were 51·8, 53·5, and 52·8. The higher values may result from the contamination of the glutamic acid spot already mentioned and a small degree of racemization taking place during hydrolysis.

There was no difference in the apparent amide content when the period of heating in 2N-hydrochloric acid varied from 1 to 6hr. Five determina-

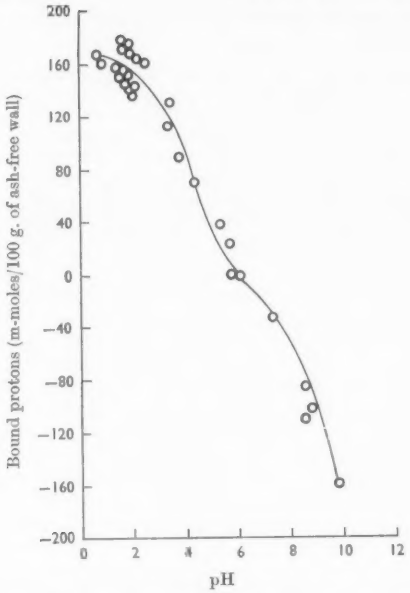

Fig. 1. Titration curve of cyst wall carried out in M-KCl. The zero on the bound proton scale has arbitrarily been set to pH 6. See the text for experimental details.

tions gave $2{\cdot}16 \pm 0{\cdot}05$ (S.D.) as the percentage of amide nitrogen in the ash-free wall. This value is equivalent to $0{\cdot}154 \pm 0{\cdot}004$ mole of amide/100 g. of material.

The titration curve is shown in Fig. 1; the substantial scatter of the points is due in part to the small quantities of material and volumes used. With the zero set at pH 6 the protons bound at pH 2 and below under conditions of high ionic strength are equivalent to the free carboxyl groups (Cannan, 1942). Thirteen points lying at or below pH 2 gave as their mean value $0{\cdot}159 \pm 0{\cdot}011$ (S.D.) mole of bound proton/100 g. of ash-free wall.

Solubilization and fractionation of wall. Phase contrast was used to examine the effect on the wall of a variety of reagents. At room temperature N-hydrochloric acid, N-sodium hydroxide, 8M-urea, 0·1M-EDTA, pH 7·9, followed by 8M-urea, cuprietheylene diamine (Coleman & Howitt, 1947), lithium isothiocyanate (50%, w/v) (Signer & Strässle, 1947), *m*-cresol and dichloroacetic acid, were without apparent action even after several days. Formic acid (98%) had some effect and the outlines of the walls became less well defined. At a concentration 1 mg./ml. in 0·05M-phosphate, pH 7·4, the enzyme from *Streptomyces griseus* (Pronase) obtained from Sigma Chemical Co. as their type V purified grade, and pancreatin supplied by the British Drug Houses Ltd., were without obvious effect after 1 week at 37°.

After oxidation with peracetic acid (Alexander & Earland, 1950) or performic acid (Thompson & O'Donnell, 1959) the wall was no longer inert; even water dissolved a considerable amount of material and after 48 hr. only thin membranes remained. A higher pH seemed to be even more effective in dissolving the oxidized wall. Conditions suitable for the complete oxidation of cystine in another insoluble protein, wool keratin (Thompson & O'Donnell, 1959), were chosen for the routine oxidation of wall and extraction was carried out at 37° by using 0·05M-sodium carbonate. Five extractions of different batches gave, on an ash-free basis, $23 \pm 3\%$ (S.D.) as the proportion undissolved.

During these experiments the weight of extract recovered plus the weight of residue was about 10% less than the original weight of material taken. Some loss was due to handling but some material might also have escaped through the bag during dialysis of the extract. From two fractionation experiments the diffusates after concentration and hydrolysis contained ninhydrin-reacting material giving a colour equivalent to 3·4 and 2·5% by wt. of the original wall extracted. After removal of salt these hydrolysates were chromatographed and contained at least 13 of the common amino acids.

Since 98% formic acid appeared to dissolve some wall material and since the performic acid used for oxidation contained excess of formic acid, in making much of the wall soluble performic acid was acting in two ways. A two-stage extraction was carried out in which wall was first treated with 98% formic acid for 16 hr. at 0° and, after freeze-drying, was extracted with 0·05M-sodium carbonate: 30, 25 and 27% of material was extracted on an ash-free basis from three preparations (extract 1). When the residues from the batches which had released 25 and 27% into solution were oxidized and extracted a further 62 and 63% respectively, based on the original weight of material, was dissolved (extract 2). In these experiments the amount extracted was taken as the difference between the amounts of ash-free starting material and ash-free residue.

Composition of wall fractions. Qualitative paper chromatography showed that all spots were present in all fractions. Extract 1 contained much less glutamic acid than did the intact wall. Cysteic acid and large quantities of glutamic acid but considerably less of the three basic amino acids were present in extract 2. The final residue contained large amounts of glutamic acid but less cysteic acid than extract 2 and was rich in the three basic amino acids.

Some of these differences are shown in the

Table 1. *Amount of some amino acids in preparations and fractions of the wall of* Colpoda steinii *cysts*

For methods of estimation and wall fractionation see the text. Several results under any one heading refer to analyses of different batches of material. Each result is the average of duplicate estimations carried out on a hydrolysate of known concentration and individual estimations did not differ from the mean by more than 5%. Results indicated by A are expressed as g. of acid from 100g. of ash-free material and those indicated by B are expressed as ninhydrin colours as a percentage of the total hydrolysate ninhydrin colour. Values under B were calculated from knowledge of the amino acid content (from chromatography) of a particular hydrolysate, the total ninhydrin colour in solution given by the same hydrolysate and the ninhydrin colour given under the same conditions by a known amount of the amino acid. For any particular acid where figures appear directly above and below each other corresponding to sub-headings A and B these figures refer to analyses on the same hydrolysate.

		Whole wall	Whole oxidized wall	Extract (1)	Extract (2)	Final residue
Glutamic acid	A	52·9 ± 0·6 (5)	51·7, 55·5, 50·5	25·3, 16·9, 23·0	57·1, 58·8	—
	B	—	37·0, 36·7	21·7, 19·1	49·9	42·9
Cysteic acid	A	—	6·4, 4·9, 6·1	—	6·1, 5·5	—
	B	—	4·4, 3·4	—	4·6	1·9
Aspartic acid	A	4·9, 6·6	5·7, 6·4, 5·5, 6·6	9·7, 9·6	3·8, 5·5	—
	B	—	4·1, 5·0	11·0	3·3	—
Glycine	A	8·9, 8·0, 8·7	—	—	—	—
	B	—	—	—	—	—

quantitative results summarized in Table 1. Two ways, explained in the legend, were chosen to express the results. The advantages of describing an amino acid content by the ninhydrin colour as a percentage of the total hydrolysate ninhydrin colour are that in addition to giving a better idea of the molar proportion of the acid it makes it possible to discuss the content of the final residue. This contained so much inorganic matter (two such fractions had non-sulphated ash values of 25 and 38%) that correction for this was almost impossible and expressing the amino acid content on a weight basis was meaningless.

The most important feature of these results is that a glutamic acid-rich fraction is not extractable as the result of treatment with formic acid but is extractable after oxidation.

DISCUSSION

The wall of the intact cyst appears to be made of several layers and although the final preparation may have contained more than one type of membrane the thick rigid layer was (by weight) the principal component.

The ash contained many elements; some, e.g. lead and tin, present in fair amounts are not likely to have had a biological origin and almost certainly arose from contamination. There is no evidence for an important degree of wall mineralization. Much of the inorganic content of the isolated material may be located in the small amounts of particulate matter which are always present. Some of these particles are probably glass fragments for small particles of glass are released into the suspension from the glass beads and container during the disruption process.

Other protozoan cyst walls which have been examined are those of an *Acanthamoeba* (Tomlinson & Jones, 1962) and *Pelomyxa illinoisensis* (Sachs, 1956), where cellulose and chitin respectively seem to be major components. The fact that *Colpoda* cyst walls are almost pure protein and very different from these other two types stresses the diversity that exists amongst Protozoa. In composition *Colpoda* cyst walls are more like bacterial spore walls, which also contain large amounts of protein (Strange & Dark, 1956; Warth, Ohye & Murrell, 1963). Small amounts of residual phosphorus were found in the preparations described here and the spore walls of some bacteria also contain residual phosphorus (Strange & Dark, 1956; Warth *et al.* 1963).

The glutamic acid content of the wall estimated by manometry was 46·5 ± 0·7g. or 0·316 ± 0·005 mole from 100g. of ash-free wall, and the aspartic acid content obtained by chromatography was 0·046 ± 0·005 mole/100g. This gives 0·360 ± 0·010 mole/100g. as the total dicarboxylic acid content, which may be compared with 0·313 ± 0·015 mole/100g., the total number of carboxyl groups present either as amide or in the free acid form. These two figures do not quite agree but the difference may not be significant and it is concluded that the bulk of the carboxylic acid side chains have been accounted for.

As judged by observation under the microscope

wall preparations appeared to resist the action of Pronase and pancreatin. This resistance may be due to the highly acidic nature of the protein, for work in this Laboratory (J. Tibbs & P. M. Whyte, unpublished work) has shown that soluble fractions of the wall and poly-α-L-glutamate are all relatively inert towards these enzymes. Resistance to proteolytic enzymes would also be expected if a substantial proportion of the glutamyl residues were peptide-bonded through their γ-carboxyl groups.

An average value, from Table 1, for the cysteic acid content of oxidized wall is 5·8g. from 100g., and this corresponds to a cystine content of 4·1g./100g. The insoluble nature of the wall appears to be largely due to the presence of disulphide bonds, for a major proportion (just over 60%) is made soluble only when these bonds are broken. That much of the glutamic acid exists as free poly-α-L-glutamate is unlikely for this substance is soluble in 0·05M-sodium carbonate. The 60% of the wall which is made soluble only when the disulphide bonds are ruptured contains about 58·0g. of glutamic acid/100g. (the average of the two values shown in Table 1) and contains about 67% of the total glutamic acid of the untreated wall preparation. This glutamic acid must be involved in direct or indirect linkage to cystine residues. It is not known whether merely glutamic acid-rich protein(s) is being dealt with or whether much of the glutamic acid exists as polyglutamate linked to a polypeptide chain of mixed amino acids through cystine residues.

When cells excyst the thick inner wall disappears and the trophic form reappears within, and finally bursts its way through, a thin, expandable, boundary membrane. If cells which are excysting are studied under phase contrast in the form of both live and crushed preparations the conclusion seems inescapable that the membrane which imprisons the trophic form at a late stage of excystment is derived from and is possibly identical with the external wall layer of the fully encysted cell. The thick inner wall appears to be digested away, and in view of the chemical nature of the preparations described in this paper, which are largely composed of this thick inner wall, this digestion should result in a supply of amino acids and in particular glutamic acid. These acids could be used for the synthesis of necessary protein and the large amount of glutamic acid could be readily deaminated to provide a source of energy.

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A New Derivative of Vitamin C and its Application to the Synthesis of Labelled Ascorbic Acid

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1. The preparation of a mono-*O*-cyclohexylidene derivative of L-ascorbic acid is described. 2. The new compound is shielded by the cyclohexanone group at C-5 and C-6 of the ascorbic acid molecule, while the double bond between C-2 and C-3 is kept intact. 3. The double bond of the new derivative is more resistant to oxidation than its parent compound. 4. Ascorbic acid is easily regenerated by mild acid hydrolysis. 5. The new derivative facilitates the synthesis of ^{14}C-labelled vitamin C.

The known instability of ascorbic acid causes marked difficulty in handling this material and has prompted us to search for a stable derivative from which the vitamin can be regenerated. Vargha (1932) reported the preparation of 5,6-mono-*O*-isopropylidene-L-ascorbic acid in 70% yield, by treatment of ascorbic acid with acetone in the presence of cupric sulphate as catalyst. The isopropylidene derivative hydrolysed in water to ascorbic acid.

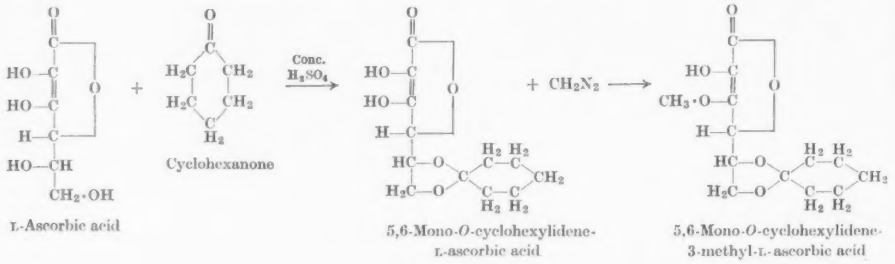
This paper describes the preparation of a new derivative of L-ascorbic acid obtained by condensation of vitamin C with cyclohexanone in the presence of catalytic amounts of strong acids. The cyclohexanone derivative is more stable than its parent compound. Nevertheless, ascorbic acid can be easily regenerated by hydrolysis with dilute acids. The new derivative was identified as 5,6-mono-*O*-cyclohexylidene-L-ascorbic acid, an analogue of 5,6-mono-*O*-isopropylidene-L-ascorbic acid.

EXPERIMENTAL

Materials. Cyclohexanone (Matheson, Coleman and Bell, East Rutherford, N.J., U.S.A.), was redistilled before use, b.p. 155°. Vitamin C (E. Merck A.-G., Darmstadt, Germany) had m.p. 188°. The $Na^{14}CN$ was prepared by zinc reduction of $Na_2{}^{14}CN_2$ (S. Von Schuching, unpublished work). L-[1-^{14}C]Ascorbic acid, specific activity 0·1 μc/mg., was obtained from L-[1-^{14}C]ascorbic acid, specific activity 7 μc/mg. (synthesized as described below), by dilution with carrier ascorbic acid.

Melting points. These were taken on a Fischer–Jones melting-point apparatus and are uncorrected.

Paper chromatography and paper electrophoresis. Paper chromatography was carried out by using the ascending technique in butan-1-ol–acetic acid–water (4:1:5, by vol.) for 18hr. on Whatman 3MM paper (Partridge & Westall, 1948; Mapson & Partridge, 1949). Paper electrophoresis was carried out as described by Bourne, Hutson & Weigel (1960) at pH5 and 10v/cm. on Whatman no. 1 paper in 0·9M-molybdate buffer for 2hr. Sorbitol was used as a



L-Ascorbic acid — Cyclohexanone — 5,6-Mono-*O*-cyclohexylidene-L-ascorbic acid — 5,6-Mono-*O*-cyclohexylidene-3-methyl-L-ascorbic acid

Scheme 1.

standard for the comparison of rates of migration (M_S). Since no indicator could be found for the universal detection of ascorbic acid and its derivatives on the paper strips, L-[1-^{14}C]ascorbic acid, specific activity 0·1 μc/mg., was used as a starting material. The migration of the compound under study was revealed by the position of radioactivity on the paper chromatogram by scanning the strip with a windowless paper-chromatogram scanner.

Assay of radioactivity. Radioactivity was determined in a Packard Tri-Carb liquid-scintillation spectrometer. The material to be analysed was dissolved in ethanol, and 0·1 ml. of the solute was added to 15 ml. of liquid-scintillation fluid, containing 4 g. of 2,5-diphenyloxazole and 0·5 g. of 2,2-*p*-phenylenebis-(5-phenyloxazole)/l. of toluene (Packard Instrument Co., La Grange, Ill., U.S.A.). A high-voltage setting of 2–600 was used. The efficiency for ^{14}C counting under these conditions was 58%, as determined with an internal standard.

Preparation and properties of 5,6-mono-O-cyclohexylidene-L-ascorbic acid (*Scheme* 1). L-Ascorbic acid (1·0 g., 5·7 m-moles) was covered with 10 ml. of redistilled cyclohexanone, and 0·14 ml. of conc. H_2SO_4 was added. The solution became clear after shaking for 10 min., and was allowed to stand for an additional 12 hr. The solution was then diluted with 50 ml. of methanol and neutralized with solid $NaHCO_3$. After removal of the precipitated Na_2SO_4 by filtration, the solution was concentrated to a small volume *in vacuo* at 65° and *n*-heptane (500 ml.) was added. The resulting precipitate was crystallized from acetone (10 ml.) and *n*-heptane (500 ml.) and recrystallized from acetone (10 ml.) and methylcylohexane (500 ml.). The yield was 1·1 g. (77%).

The cyclohexylidene derivative of ascorbic acid is freely soluble in light petroleum and water, and insoluble in *n*-heptane and methylcyclohexane.

The new compound had m.p. 185° (decomp.) and $[\alpha]_D$ +37·0° (*c* 2 in methanol) (Found: C, 56·1; H, 6·4. $C_{12}H_{16}O_6$ requires C, 56·2; H, 6·2%).

A sample (0·25 g., 1·0 m-mole) of this material was dissolved in 100 ml. of water and a portion titrated with 0·1 N-NaOH. It required exactly 1 equiv. of the alkali (with phenolphthalein as indicator). The formation of the monosodium salt corresponds to the neutralization of the enol group of ascorbic acid at C-3 (Rosenberg, 1942, p. 295).

The monosodium salt gave an intense blue colour with aq. $FeCl_3$, indicating another free enol group at C-2 (Reichstein & Oppenauer, 1934).

Titration of a sample with indophenol dye (Rosenberg, 1942, p. 316) required 1 equiv. of the reagent, which proved that the double bond is retained. No change in titre was observed after passing O_2 from a tank through a 5% solution of the monocyclohexylidene derivative in water, which indicates an increased stability of the derivative.

The following studies were carried out with derivatives prepared from L-[1-^{14}C]ascorbic acid, specific activity 0·1 μc/mg.

Paper chromatography of the new compound revealed a spot with R_F 1·0 (R_F for ascorbic acid is 0·37). During paper electrophoresis, the substance migrated towards the anode, the M_S value being 0·79 (M_S for ascorbic acid is 1·53).

L-[1-^{14}C]*Ascorbic acid.* A sample (1 g., 3·9 m-moles) of 5,6-mono-*O*-cyclohexylidene-L-[1-^{14}C]ascorbic acid, specific activity 0·1 μc/mg., was covered with 50 ml. of 10% formic acid. The progress of hydrolysis was followed by paper chromatography, and was complete after 24 hr. at room temperature. The formic acid solution was evaporated to dryness and the residue washed with *n*-heptane to remove cyclohexanone. The residue was next dissolved in ethanol (15 ml.), and peroxide-free ether (150 ml.) was added. Some flocculent material was filtered off and the ether–ethanol solution was concentrated to 1·0 ml. and stored at 4°. The resulting crystals weighed 490 mg. (77% yield), m.p. 186°. The peak of radioactivity coincided with the position of unlabelled ascorbic acid on a paper chromatogram.

5,6-Mono-O-cyclohexylidene-3-methyl-L-ascorbic acid (*Scheme* 1). An ether suspension of 5,6-mono-*O*-cyclohexylidene-L-[1-^{14}C]ascorbic acid (2·0 g., 7·8 m-moles), specific activity 0·1 μc/mg., was cooled to 0° and an excess of diazomethane in cold ether was added (Fieser, 1955). The mixture was allowed to come to room temperature and a slow evolution of N_2 took place. The ether and excess of diazomethane were removed by spontaneous evaporation, leaving an oily residue that resisted all attempts at crystallization. The oily residue was dried thoroughly; it had no boiling point but decomposed at 150–160°. The ester did not reduce 2,6-dichlorophenol-indophenol; it did not migrate during paper electrophoresis, but moved on a paper chromatogram (R_F 1·0). The presence of a methoxy group was shown by boiling 1 g. of the material with an excess of hydriodic acid and collecting the resulting methyl iodide in ethanolic $AgNO_3$ (Richter-Anschütz, 1928). Without prior neutralization a blue colour was obtained with $FeCl_3$, indicating a free enol group at C-2 of the ascorbic acid molecule and a methyl group at C-3. Analysis corresponded to the addition of 1 methyl group/mol. (Found: C, 57·1; H, 6·8. $C_{13}H_{18}O_6$ requires C, 57·7; H, 6·8%).

Synthesis of L-[1-^{14}C]ascorbic acid with 5,6-mono-O-cyclohexylidene-L-ascorbic acid as an intermediate. L-Xylosone (1·2 g., 8 m-moles) was condensed with Na^{14}CN (0·294 g., 6 m-moles, 10 mc of ^{14}C) according to the procedure of Salomon, Burns & King (1952), but no carrier ascorbic acid was added before hydrolysis of the imino-L-ascorbic acid. The solution after hydrolysis was evaporated *in vacuo* at 40°. The syrup was thoroughly dried at room temperature at high vacuum. Then 10 ml. of redistilled cyclohexanone and 0·14 ml. of conc. H_2SO_4 were added to the residue and the mixture was shaken for 10 min. and kept at room temperature for another 12 hr. Ethanol (50 ml.) was added and also enough $NaHCO_3$ to neutralize the solution. The resulting salts were filtered off, the solution was concentrated *in vacuo* at 65° to 10 ml. and *n*-heptane (500 ml.) was added. After 24 hr. at −20°, 5,6-mono-*O*-cyclohexylidene-L-[1-^{14}C]ascorbic acid settled partly at the bottom of the flask as a sticky mass, while another part floated in the heptane layer. The *n*-heptane layer was filtered and the precipitates were combined. Further purification was carried out by reprecipitation from acetone and *n*-heptane and also from acetone and methylcyclohexane. Separation from inorganic ions, which interfere with the crystallization of ascorbic acid, is thus achieved. The air-dried material was hydrolysed to free ascorbic acid with 10% formic acid as described above. L-[1-^{14}C]Ascorbic acid in water was further purified by passage over a Duolite-A-4 resin column (Chemical Process Co., Redwood City, Calif., U.S.A.) in the acetate form (column 2 cm. × 20 cm., resin bed 10 cm.).

The eluate was evaporated, taken up in 30 ml. of ethanol and 300 ml. of peroxide-free ether was added. A flocculent

precipitate was filtered off and the ethanol–ether solution concentrated to a small volume and kept at 4°; 510 mg. of L-[1-^{14}C]ascorbic acid, specific activity 7 μc/mg. (36% radiochemical yield), was obtained, m.p. 186°. A mixed m.p. with an authentic sample of L-ascorbic acid showed no depression. Radioactive purity was established by scanning a paper chromatogram with a windowless scanner. A single peak coincided with the position of unlabelled ascorbic acid on the paper strip.

An alternative procedure for hydrolysing the derivative to ascorbic acid consists in stirring monocyclohexylidene-L-ascorbic acid with a tenfold excess of Dowex-50 (H^+ form) for 1 hr. in 50 ml. of water. After the resin had been filtered off and the solution evaporated, L-ascorbic acid was obtained in crystalline form. This eliminates the use of an anion-exchange column in the acetate form which was previously employed in preparing ^{14}C-labelled ascorbic acid.

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The Effect of Ultraviolet Irradiation on Collagen-Fold Formation

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1. A study has been made of the effect of ultraviolet irradiation, in the presence of air and nitrogen, on the conformational changes taking place in cooled solutions of gelatin prepared from thermally denatured neutral-salt-soluble collagen. 2. The increase in negative rotation and viscosity at 15° for irradiated and thermally denatured solutions of collagen becomes less as the irradiation time is increased. 3. The principal effect of ultraviolet irradiation is the fission of the primary collagen peptide chains, eventually yielding chain lengths incapable of stabilizing a helical structure. 4. Irradiation in both air and nitrogen results in a loss of tyrosine, histidine and phenylalanine, with more denaturation occurring in the presence of nitrogen.

Ultraviolet irradiation has been shown to modify proteins in general (McLaren, 1949; Rideal & Roberts, 1951; McLaren & Shugar, 1964) and collagen in particular (Cooper & Davidson, 1965; Nishigai, 1964). It was therefore decided to study the action of ultraviolet irradiation on the conformational changes induced in solutions of neutral-salt-soluble collagen on cooling. The conditions governing this coil-to-helix transition in cooled solutions of gelatin have been studied in detail (Harrington & Hippel, 1961; Hippel & Wong, 1962, 1963*a*,*b*; Piez & Carrillo, 1964; Mandelkern & Stewart, 1964; Kuhn, Engel, Zimmermann & Grassmann, 1965).

MATERIALS AND METHODS

Preparation of neutral-salt-soluble collagen. The purified neutral-salt-soluble collagen from calf skin was the same as that used in a previous publication, where the extraction and purification was described in detail (Cooper & Davidson, 1965).

Ultraviolet irradiation. Collagen solutions were prepared in 0·15M-potassium acetate buffer, pH 4·8, which is known to have the minimum effect on mutarotation (Piez & Carrillo, 1964). These solutions were irradiated with a Hanovia UVS 220A lamp, operated by a regulated power supply, at a solution temperature of 0–4° in a cold room. The lamp used is rated to give an ultraviolet intensity at 92cm. of 420–540μw/cm.^{2} of irradiation below 4000Å. The solutions (10ml.) were placed in fused quartz test tubes at a distance of 46cm. from the lamp. Irradiations were either in the presence of air or oxygen-free nitrogen, the latter containing not more than 10v.p.m. of oxygen and no oxides of nitrogen or carbon. In the latter case the nitrogen, after passing through a solution of alkaline pyrogallol and a spray trap, was then bubbled through the protein solution for 30min. before irradiation was commenced, and during the whole period of irradiation. The bubbling rate was about 100ml./min. and was kept fairly low to avoid excessive frothing of the solution.

Viscosity. Viscosities were measured at 15±0·05° as described previously (Cooper & Davidson, 1965).

Optical Rotation. Optical rotation was measured at 15±0·07° in a 40cm. water-jacketed tube by using a Bellingham and Stanley polarimeter equipped with a sodium lamp.

The procedure for following helix formation was to irradiate the collagen solution for a given period at 0–4°, heat-denature at 40° for 10min. and then cool for 45sec. under tap water. A test sample was then immediately transferred to the viscometer or to the polarimeter tube kept at 15°, and readings were taken at regular intervals for periods up to 48hr.

Amino acid analysis. Amino acid composition was determined with a Spinco amino acid analyser after hydrolysis with 6N-HCl under reflux for 24hr.

Ultracentrifugation. The method was the same as that used previously (Cooper & Davidson, 1965).

Spectrophotometry. Absorption curves were measured in 10mm. quartz cells in a Beckman model DB recording spectrophotometer.

RESULTS

Optical rotation and viscosity. In the first series of experiments, solutions of neutral-salt-soluble collagen (1·36mg./ml.) were irradiated in the presence of air for periods of up to 19hr., denatured at 40° for 10min., and the helix formation was followed by optical rotation while the solution was kept at 15° (Fig. 1). The results for a non-irradiated solution after thermal denaturation are included. Here a recovery of 59% of the helical content of

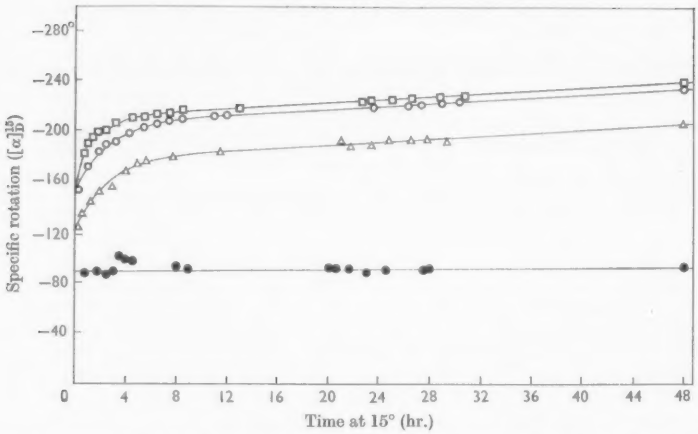

Fig. 1. Specific rotation ($[\alpha]_D^{15}$) as a function of time for solutions of neutral-salt-soluble collagen (1·36 mg./ml.) irradiated with ultraviolet light at 0–4°, heat-denatured at 40° and then kept at 15°. □, Not irradiated; ○, irradiated for ½ hr.; △, irradiated for 3 hr.; ●, irradiated for 19 hr.

Table 1. *Rate of collagen-fold formation after ultraviolet irradiation and cooling to* 15°

Solutions were irradiated at 0–4° for the times shown, or not irradiated, and then thermally denatured at 40° for 10 min., before being cooled to 15°.

Material	Initial rate of mutarotation at 15° $(d[\alpha]_D/dt)_0$ (deg./min.)	$10^{-2} \times$ Initial rate of viscosity recovery at 15° $(d\eta/dt)_0$ (dl./g. min.)
Neutral-salt-soluble collagen	0·70	2·5
Neutral-salt-soluble collagen after ½ hr. irradiation	0·46	1·6
Neutral-salt-soluble collagen after 3 hr. irradiation	0·36	0·3
Neutral-salt-soluble collagen after 19 hr. irradiation	0	0

the native collagen was obtained, which is in agreement with reported values (50–86%; Harrington & Hippel, 1961). It is evident from these data (Fig. 1 and Table 1) that ultraviolet irradiation decreases the initial rate of mutarotation, i.e. $(d[\alpha]/dt)_0$, and considerably decreases the amount of helix formation in the partial re-formation on cooling of the collagen-fold or some modification of this structure (Harrington & Hippel, 1961; Veis, 1964). Further, with irradiation for about 19 hr., both the recovery of helical nature and the association of sub-unit chains (see below) virtually cease to take place. The specific rotation is decreased to a $[\alpha]_D^{15}$ value of −89°, which corresponds to the rotation of α-helix-forming proteins and collagen in the denatured form ($[\alpha]_D \simeq -90°$ to −120°; Harrington & Hippel, 1961), indicating complete loss of the poly-L-proline II-type helix.

A parallel study was made of the change in viscosity at 15° (Fig. 2 and Table 1). The irradiation caused a decrease in the initial rate of viscosity recovery, and the amount of chain association occurring parallel with the regain in helical content.

Fig. 3 illustrates the relationship between reduced viscosity on cooling for 48 hr. at 15°, which is a measure of aggregation or inter-chain association (Harrington & Hippel, 1961), and irradiation time, which is directly related to irradiation damage. This is not linear, and the main effect of irradiation is almost complete in the first 3 hr. under the present experimental conditions. Thus the first breaks in the polypeptide chain are clearly more effective than the later ones in reducing sub-unit chain association as measured by viscosity. The relation is also presented (Fig. 3) between specific rotation, which is directly related to helical content or intra-chain reaction (Hippel & Wong, 1963*a*,*b*), and irradiation time. This is a linear relationship.

Hippel & Wong (1963*b*) found a similar relation between $\Delta[\alpha]_{313}$ (the difference between the specific rotation at the high- and low-temperature ends of the helix-to-coil transition) and the number of chain breaks brought about by various enzymes. They also found that the initial rate of mutarotation decreases as the chain length is diminished, and

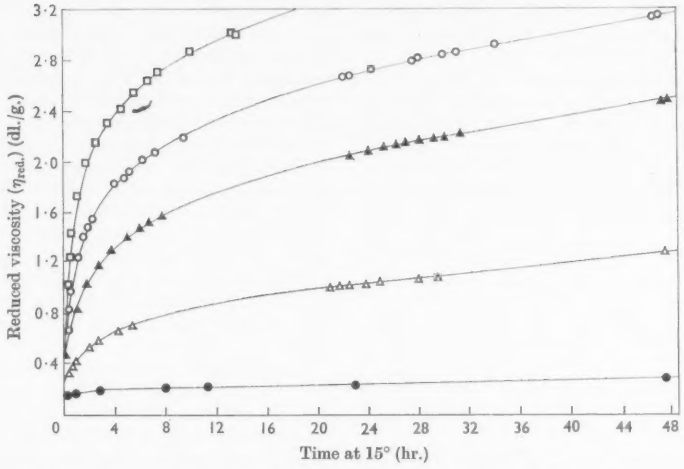

Fig. 2. Reduced viscosity ($\eta_{red.}$) as a function of time for solutions of neutral-salt-soluble collagen (1·36 mg./ml.) irradiated with ultraviolet light at 0–4°, heat-denatured at 40° and then kept at 15°. □, Not irradiated; ○, irradiated for ½ hr.; ▲, irradiated for 1 hr.; △, irradiated for 3 hr.; ●, irradiated for 19 hr.

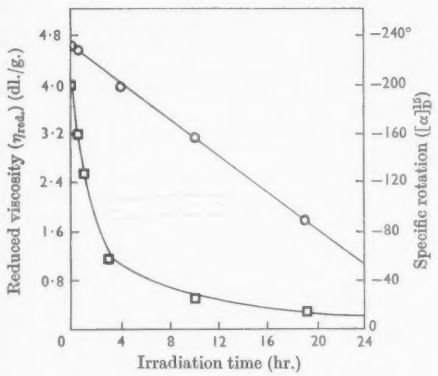

Fig. 3. Specific rotation (○) and reduced viscosity (□) as functions of irradiation time at 0–4°, for solutions of neutral-salt-soluble collagen (1·36 mg./ml.) irradiated with ultraviolet light, heat-denatured and then kept at 15° for 48 hr.

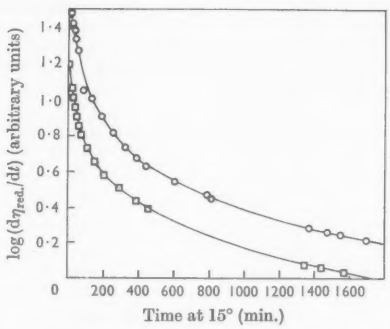

Fig. 4. Plots of log ($d\eta_{red.}/dt$) as a function of time at 15°, for solutions of neutral-salt-soluble collagen (1·36 mg./ml.) after heat-denaturation at 40° (○), and after ultraviolet irradiation for 1 hr. followed by heat-denaturation at 40° (□).

that the first breaks in the chain are more effective than later ones in decreasing the initial rate of mutarotation. This is also apparent from the present data (Figs. 1 and 3), if the irradiation time is taken as being proportional to the number of breaks in the chain, which is presumably a reasonable assumption in the initial stages of irradiation.

To determine the order of reaction for recovery of optical rotation and viscosity at 15°, with and without irradiation, log ($d[\alpha]/dt$) and log ($d\eta_{red.}/dt$) were plotted against t. Fig. 4 illustrates a typical relationship. Except possibly for the first hour of recovery time, the period during which readings were most difficult to measure accurately as the reaction rate was high, these relationships were non-linear, showing that the re-formation reaction

in terms of helical formation and aggregation is in the main not a first-order reaction (Harrington & Hippel, 1961; Piez & Carrillo, 1964).

The accumulative effect of the irradiation, which also shows that the irradiation damage is not reversible, is illustrated in Fig. 5. In this experiment a solution of neutral-salt-soluble collagen (1·36mg./ml.) was irradiated for 30min., heat-denatured at 40° for 10min. and kept at 15° for 48hr. while the viscosity was measured at regular intervals. After this the solution was immediately irradiated again for a further 30min., heat-denatured at 40° for 10min. and kept at 15° for 32hr., while the viscosity was again recorded. Fig. 5 shows that after the first irradiation and 32hr. re-formation time at 15° the reduced viscosity was 2·88dl./g., whereas the reduced viscosity after the second irradiation and the same time for re-formation was 1·64dl./g. For a continuous irradiation interval of 60min. with 32hr. re-formation time the reduced viscosity was 2·25dl./g. (Fig. 2). The latter is not a strict comparison since the reduced viscosity had not reached equilibrium even after 48hr. It has been reported (Hippel & Harrington, 1959; Piez & Carrillo, 1964) that viscosity can increase over a period of many days.

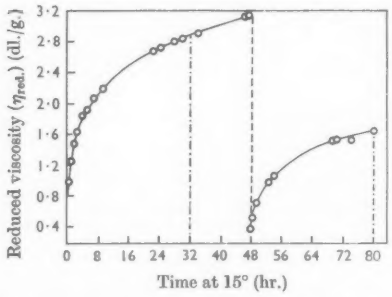

Fig. 5. Reduced viscosity ($\eta_{red.}$) as a function of time for a solution of neutral-salt-soluble collagen irradiated with ultraviolet light at 0–4° for ½hr., heat-denatured at 40° and then kept at 15° for 48hr. The same solution was then irradiated for a further ½hr., heat-denatured at 40° and then kept at 15° for 32hr.

Experiments were then conducted to follow the differences in helix formation and aggregation brought about by ultraviolet irradiation in air and nitrogen. These data are illustrated graphically in Figs. 6 and 7, where samples of the same collagen solution were irradiated for ½hr. and 1hr. in air and with pure oxygen-free nitrogen bubbling through the solution. The collagen, when irradiated in the presence of nitrogen, gave less recovery of optical rotation and viscosity at 15° than when the irradiation was done in the presence of air. Fig. 6 shows that irradiation for 1hr. in air actually gives a greater recovery of reduced viscosity than ½hr. irradiation in the presence of nitrogen.

A summary is given in Table 2 of the reduced

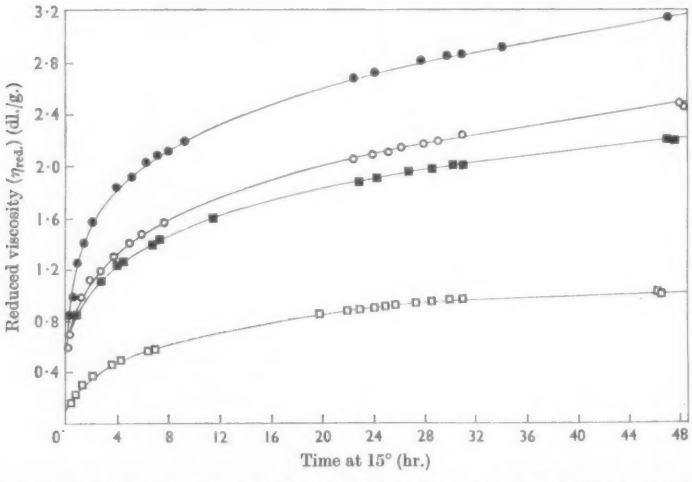

Fig. 6. Reduced viscosity ($\eta_{red.}$) as a function of time at 15°, for solutions of neutral-salt-soluble collagen (1·36mg./ml.) irradiated in air and nitrogen with ultraviolet light, heat-denatured at 40° and then kept at 15°. ●, Irradiated for ½hr. in air; ○, irradiated for 1hr. in air; ■, irradiated for ½hr. in nitrogen; □, irradiated for 1hr. in nitrogen.

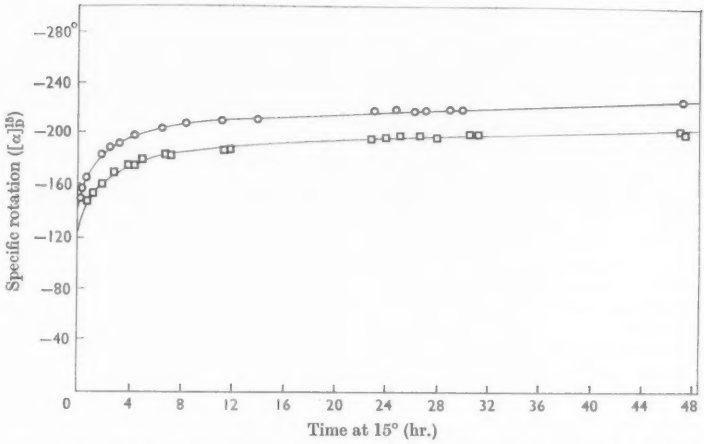

Fig. 7. Specific rotation ($[\alpha]_D^{15}$) as a function of time at 15°, for solutions of neutral-salt-soluble collagen (1·36 mg./ml.) irradiated with ultraviolet light for ½ hr. in air (○) and ½ hr. in nitrogen (□), followed by heat-denaturation at 40°.

Table 2. *Summary of reduced viscosity and specific rotation values for neutral-salt-soluble collagen subjected to ultraviolet irradiation*

Values were obtained after keeping solutions at 15° for 48 hr.

Treatment	Reduced viscosity (dl./g.)	Specific rotation ($[\alpha]_D$)
Control: no irradiation and no heat-denaturation	15·94	−339°
Heat-denaturation but no irradiation	4·10	−235
Irradiated for ½ hr. in air with heat-denaturation	3·16	−230
Irradiated for 1 hr. in air with heat-denaturation	2·47	—
Irradiated for 3 hr. in air with heat-denaturation	1·30	−204
Irradiated for 10 hr. in air with heat-denaturation	0·55	−159
Irradiated for 19 hr. in air with heat-denaturation	0·28	−89
Irradiated for ½ hr. in N_2 with heat-denaturation	2·20	−202
Irradiated for 1 hr. in N_2 with heat-denaturation	1·02	—

Table 3. *Amino acid composition of ultraviolet irradiated neutral-salt-soluble collagen*

Neutral-salt-soluble collagen was irradiated in nitrogen for 19 hr. before analysis.

Amino acid	Amino acid composition (g./100 g. of protein)
Ala	8·59
Arg	8·43
Asp	5·20
Glu	11·15
Gly	20·15
His	0·33
Hyl	1·15
Hyp	9·55
Ile	1·65
Leu	3·30
Lys	3·90
Met	0·77
Phe	1·07
Pro	11·52
Ser	3·48
Thr	2·06
Tyr	0·33
Val	2·45
Amide N	0·91
Total	95·08

viscosity and specific rotation obtained for solutions of neutral-salt-soluble collagen kept at 15° for 48 hr., after various irradiation treatments.

Amino acid analysis. In Table 3 the amino acid analysis of neutral-salt-soluble collagen after irradiation in the presence of nitrogen is given. Comparison of these values with those reported (Cooper & Davidson, 1965) for neutral-salt-soluble collagen

in the native form, and after ultraviolet irradiation in air, shows that the only amino acids partially destroyed by the irradiation were histidine, phenylalanine, methionine and tyrosine. The greatest losses are apparent after irradiation in nitrogen.

Ultracentrifugation. Ultracentrifugation of native neutral-salt-soluble collagen has been shown to give two major components (S_{35} 3·48 and 4·50s), corresponding to the α- and β-components of collagen (Cooper & Davidson, 1965). After the irradiation in air one main heterogeneous component (S_{35} 2·29s) was obtained (Cooper & Davidson, 1965), whereas after irradiation in nitrogen a similar heterogeneous component with a lower sedimentation coefficient (S_{35} 1·84s) was found.

Reaction with 2,4-dinitrophenylhydrazine. Gallop (1964) has proposed a structure for the α-components of tropocollagen based on four peptide chains linked end-to-end through a hexose residue, which is adjacent to aspartic acid on one of the chains and tyrosine on the other (see Fig. 8 in Gallop, 1964). Since ultraviolet irradiation destroys about half the tyrosine in collagen this should, through steric considerations, make the hexose, if present, more readily available for reaction with the above reagents.

Accordingly, solutions of neutral-salt-soluble collagen were tested: (*a*) in the native form; (*b*) after heat-denaturation for 10min. at 40°; (*c*) after irradiation for 3 and 19hr., followed by heat denaturation. Solutions of the same initial collagen concentration (1·36mg./ml.) were dialysed, and then an equal volume of 0·006% 2,4-dinitrophenylhydrazine in N-hydrochloric acid was added, the reaction being allowed to proceed at 25° for 5min. The spectra in the range 360–420mμ were immediately measured. The results (Fig. 8) show that the irradiated solutions (*c*) gave significantly higher extinctions than the two control solutions (*a* and *b*).

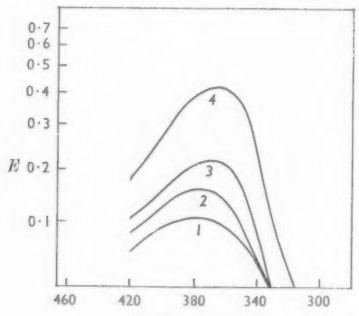

Fig. 8. Extinction curves for neutral-salt-soluble collagen (1·36mg./ml.) treated with 2,4-dinitrophenylhydrazine at 25°. *1*, Collagen in the native form; *2*, collagen heat-denatured at 40°; *3*, collagen irradiated with ultraviolet light for 3hr. and heat-denatured at 40°; *4*, collagen irradiated with ultraviolet light for 19hr. and heat-denatured at 40°.

DISCUSSION

The main factors that control the rate and extent of helix formation into the unique collagen-type triple helix in solutions of cooled gelatin are the pyrrolidine ring content, due to the presence of proline and hydroxyproline, the relative content of the α-, β- and γ-components, and the solvent employed (Piez & Carrillo, 1964). According to Harrington & Hippel (1961) the following three-stage mechanism occurs in the re-formation of the collagen-type structure: (1) The pyrrolidine-rich portions of the peptide chain undergo initial changes that 'nucleate' the poly-L-proline II-type helix. (2) This helix structure propagates outwards from these nuclei along single gelatin chains (Flory & Weaver, 1960). (3) The formation of the unique collagen-fold along individual chains makes possible lateral chain association through inter-chain hydrogen bonding (Veis, 1964). The second step may be monitored by changes in optical rotation and the last by the increase in viscosity.

Since the current experiments have shown that ultraviolet irradiation affects the rate and extent of helix formation, it is necessary to consider which of the above factors are affected by the irradiation. The amino acid analyses show that no significant decrease in the hydroxyproline and proline content occurs after irradiation, and therefore the initial nucleation step involving the pyrrolidine ring cannot be affected by irradiation. The amino acid analyses also show that, apart from histidine, phenylalanine and tyrosine, which are present in small amounts, no great loss of amino acids occurs on irradiation. As reported previously (Cooper & Davidson, 1965), the main effect of irradiation is the conversion of the α- and β-components into smaller peptide chains. Therefore the effect of ultraviolet irradiation on collagen-fold formation would appear to be due to the formation of shorter peptide chains, in the random-coil form, which hinder the propagation of the helical structure starting at the pyrrolidine rings and proceeding along the single-chain random coils (Hippel & Wong, 1963*b*). This would presumably also decrease the amount of lateral chain association between single helical chains, and any single chain folding giving helices stabilized by intramolecular association (Veis, 1964). Both these would result in a diminished recovery of viscosity on cooling.

Hippel & Wong (1963*b*) found that the degree of helical recovery taking place on cooling decreased

markedly as the molecular weight of the gelatin in solution was decreased by enzyme action. They estimate that a minimum chain length of 40–80 residues is required to generate stable elements of the collagen-type helix, and that the final helical content is independent of the method of breaking the peptide chain. The current amino acid analyses show losses of tyrosine, histidine, phenylalanine and methionine for the irradiation of collagen in air. These losses correspond to the destruction of about 11 amino acid residues/1000 residues, which is equivalent to about 11 breaks/α-chain of mol.wt. 100000. Hippel & Wong (1963*b*) have shown that no helical content is found in cooled solutions of gelatin after enzymic reaction causes about 40 breaks/polypeptide chain of mol.wt. 100000.

As shown above the initial rates of mutarotation and viscosity recovery are decreased by irradiation, as are the final specific rotation and reduced viscosity attained after cooling at 15° for 48hr. The initial breaks in the polypeptide chain brought about by irradiation are also more effective in decreasing these initial rates than the later ones. These results are in agreement with those found by Hippel & Wong (1963*b*) for chain-fission brought about by enzymes, and confirm the conclusion that the results are independent of the method of fission.

In a previous paper (Cooper & Davidson, 1965) it was concluded that the coil-to-helix transition in solutions of gelatin, prepared by the ultraviolet irradiation of neutral-salt-soluble collagen for 19hr., followed by storing at 0°, could be one of the factors accounting for the abnormally high molecular weights obtained by gel filtration at 20°. The present results show, however, that after 19hr. of irradiation the same gelatin preparation had a very low helical recovery on cooling to 15°.

Comparison of irradiation in air and nitrogen shows that in the latter case the amount of collagen-fold re-formation is considerably decreased for the same irradiation times. The amino acid analyses indicate a larger decrease in the histidine and phenylalanine content after irradiation in nitrogen. In addition, the sedimentation coefficient after irradiation in nitrogen is lower. These results, in conjunction with the viscosity and optical-rotation data, show that more denaturation occurs in the presence of nitrogen than in air. Generally, it appears that irradiation of proteins in the presence of nitrogen results in less denaturation when compared with irradiation in air, and may in fact produce aggregation (see, e.g., Rideal & Roberts, 1951; Marsh, 1964). Therefore the present findings require further investigation to elucidate the effects of these two reaction media.

Some support for the theory (Gallop, 1964) that the α-components of collagen consist of four peptide chains linked end-to-end through hexose molecules was obtained from the increased reaction of gelatin, prepared from irradiated collagen, with 2,4-dinitrophenylhydrazine.

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Role of the Intestinal Brush Border in the Absorption of Cholesterol in Rats

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1. Short-term incubation of the everted intestinal sacs of rats in media containing cholesterol oleate or cholesterol plus oleic acid resulted in rapid hydrolysis, but no synthesis, of the sterol ester. 2. On separation of the brush border from the rest of the mucosal cell, almost all of the hydrolytic activity and appreciable amounts of the synthetic activity of the whole cell were found to be present in the brush-border fraction. 3. The isolated brush-border fraction contained considerable amounts of cholesterol, which was always present in the unesterified state; the rest of the cell contained about an equal amount of unesterified cholesterol, but, in addition, small but definite amounts of the esterified sterol were also found in this fraction. 4. On feeding rats with [4-^{14}C]cholesterol, which was diluted with 3mg. of cholesterol, it was found that the brush border very rapidly took up the fed sterol without changing its net content of cholesterol. No traces of radioactive cholesterol ester could ever be detected in the isolated brush border after feeding with ^{14}C-labelled esterified or unesterified cholesterol. 5. The appearance of the labelled sterol was quite rapid in the rest of the cell also, where small proportions were found in the esterified state. 6. Therefore the sequence of events in the absorption of cholesterol appears to be: the dietary cholesterol esters are hydrolysed by the cholesterol ester hydrolase of pancreas or of the mucosal brush border or both, after which the brush border rapidly absorbs the de-esterified sterol and transfers it into the mucosal cell, by a mechanism of displacement, where it is slowly re-esterified for transport through the lymph.

Several studies on the mechanism of absorption of cholesterol have established that the sterol can enter the mucosal cells only as unesterified cholesterol (Friedman, Byers & St George, 1956; Treadwell, Swell & Vahouny, 1962) and that the absorbed sterol appears in the lymph largely as its ester (Swell, Trout, Hopper, Field & Treadwell, 1959; Treadwell, Swell, Vahouny & Field, 1959). However, little is known about the fate of the absorbed sterol inside the mucosal cell before it eventually appears in the lymph. Nevertheless, the hydrolysis of cholesterol esters and the subsequent esterification of most of the free cholesterol seem to be essential steps in the overall process of absorption of cholesterol esters, and it has been demonstrated that both pancreas and intestinal mucosa of rats contain both cholesterol ester hydrolase and synthetase (Murthy, Mahadevan & Ganguly, 1961*a*; Murthy, Mahadevan, Seshadri Sastry & Ganguly, 1961*b*; Murthy & Ganguly, 1962). It is also known that the mucosal enzymes are not of pancreatic origin and are much less active than the pancreatic enzymes (Murthy *et al.* 1961*b*; Murthy & Ganguly, 1962; Lossow, Migliorini, Brot & Chaikoff, 1964).

The role of the brush border in the absorption of cholesterol is not clearly understood. By isolating the brush border of rat intestine, we have shown that it contains almost all of the hydrolytic activity of the mucosal cells and that, during the absorption of cholesterol, it rapidly takes up the de-esterified sterol and transfers it into the mucosal cell, where it is re-esterified.

We had previously shown that the hydrolytic and synthetic activities of rat pancreas towards cholesterol esters are due to two separate enzymes (Murthy & Ganguly, 1962). By separating the brush border from the rest of the mucosal cell, we have demonstrated a differential distribution of the two enzymes of rat-intestinal mucosa.

MATERIALS AND METHODS

[4-^{14}C]Cholesterol was obtained from The Radiochemical Centre, Amersham, Bucks. [4-^{14}C]Cholesterol stearate and oleate were prepared enzymically with acetone-dried goat

pancreas as the source of enzyme and under the conditions described by Murthy & Ganguly (1962), who also give the sources of the other materials used.

Normal male albino rats of this Institute strain, weighing about 150g., were used in all experiments.

The procedure of Miller & Crane (1961) was adopted for the isolation of the brush border of rat-intestinal mucosa. Miller & Crane (1961) used the term 'supernatant' to mean the mucosal cell stripped of the brush border, and we have used the same terminology here. These workers had shown that 75–86% of the total invertase (Miller & Crane, 1961) and 68–75% of the total alkaline phosphatase (Holt & Miller, 1962) of the intestinal mucosa of hamster are localized in the brush-border fraction. When we assayed the invertase according to Miller & Crane (1961) and alkaline phosphatase according to Heppel (1955), in the supernatant and brush-border fractions obtained from rat-intestinal mucosa, we found that about 75% of the total invertase activity of the intestinal mucosa could be recovered in the brush border, the rest being present in the supernatant; we found that, on first sedimentation after the separation of the brush border from the mucosal cell, about 55–60% of the total activity of the alkaline phosphatase was recovered in the brush-border fraction. But during the course of purification, which was carried out by successive washing of the brush-border fraction with ice-cold 5mM-EDTA, most of the alkaline phosphatase was released from the sediment into the washing medium, which did not occur with the invertase. When examined under the phase-contrast microscope, our preparations resembled the description of the isolated brush border given by Miller & Crane (1961).

In feeding experiments the rats were first starved for 24hr., after which they were given 0·4μc of [4-^{14}C]cholesterol diluted with 3mg. of cholesterol and dissolved in 0·2ml. of groundnut oil. Labelled cholesterol stearate was administered in a similar manner after diluting it with 3mg. of cholesterol stearate. At suitable time-intervals the animals were killed by heart puncture under light ether anaesthesia and the whole intestine was removed into beakers maintained at 0°. The intestinal contents were immediately washed out with ice-cold 0·9% NaCl, and the mucosa was scraped off with the blunt end of a scalpel. The brush border was then separated from the rest of the mucosa according to the procedure of Miller & Crane (1961).

The cholesterol ester-hydrolysing and -synthesizing activities of the mucosal brush border and of the supernatant fractions, as well as of the 0·25M-sucrose homogenates of the whole mucosa, were assayed by the method of Murthy & Ganguly (1962).

In experiments in which everted sacs were used, the whole intestine of each individual rat was cut into five segments, each approx. 18cm., which were weighed and then everted as described by Wilson & Wiseman (1954). The five segments from each rat were incubated in separate flasks containing 30ml. each of the same reaction mixture. Unless otherwise stated, the reaction mixture for the cholesterol ester-synthesizing activity consisted of 30ml. of 0·1M-veronal buffer, pH6·1, 30μmoles of cholesterol, 60μmoles of oleic acid and 120μmoles of sodium taurocholate; for the cholesterol ester-hydrolysing activity it was 30μmoles of cholesterol oleate and 120μmoles of sodium taurocholate in 30ml. of 0·1M-veronal buffer, pH8·6. The substrates (cholesterol plus oleic acid or cholesterol oleate) were first dissolved in ethanol and then dispersed in the respective buffers to give a final concentration of 10% of ethanol in the media. The serosal medium for the synthetic activity was 0·1M-veronal buffer, pH6·1, and for the hydrolytic activity it was 0·1M-veronal buffer, pH8·6. The incubations were carried out with constant shaking in a metabolic shaker, which was maintained in a water bath at 37°. At the desired time-intervals 2ml. portions of the incubation medium were withdrawn from each flask and pooled for subsequent analysis.

Extraction and chromatographic separation of cholesterol and its esters, and their radioactivity and chemical measurements, were carried out as described by Murthy, David & Ganguly (1963). Proteins were estimated by the biuret reaction (Gornall, Bardawill & David, 1949).

RESULTS

We had demonstrated in our earlier work that the intestinal mucosa of rats contains both the hydrolytic and synthetic activities of cholesterol esterases (Murthy *et al.* 1961*b*; Murthy & Ganguly, 1962). Fig. 1 shows that incubation of the everted sacs with cholesterol oleate in the medium resulted in rapid hydrolysis of this ester, the rate of hydrolysis being linear up to 30min., after which time the activity had distinctly slowed down, but continued up to 20hr. This hydrolytic activity, when

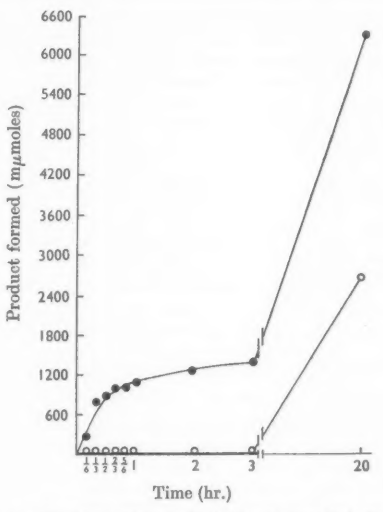

Fig. 1. Cholesterol ester hydrolysis and synthesis by the everted intestinal sacs. The experimental conditions are described in the Materials and Methods section. No perceptible esterification of the medium cholesterol took place at the earlier time-intervals, as can be seen from the points (○) on the base line. ●, Hydrolysis of cholesterol oleate; ○, synthesis of cholesterol oleate.

expressed in terms of mμmoles of cholesterol liberated/g. of intestine/hr., was 1800, 630, 460 and 315 at ½, 2, 3 and 20hr. respectively. In striking contrast with the very rapid hydrolysis of the ester present in the medium there was no esterification of the free sterol of the medium even after 3hr. incubation; however, on prolonging the incubation to 20hr. some esterification took place.

Although the everted sacs could not effect any esterification of the cholesterol of the medium at the earlier time-intervals of incubation, it is shown in Table 1 that rapid synthesis of the sterol ester can be demonstrated if the mucosal cells are broken by homogenization. The sterol-esterifying activity, as manifested by the everted sacs only after prolonged incubation (Fig. 1), is therefore probably due to the disruption of the mucosal cells caused by such an incubation, which might result in the release of the hitherto inaccessible esterifying enzyme. The rapid hydrolysis of the medium ester by the everted sacs, on the other hand, made it obvious that the hydrolytic enzyme is in intimate contact with the substrate. One possibility is that the hydrolytic enzyme is situated on the brush border. That this is indeed so is shown in Table 2, where it is demonstrated that the brush border, when separated from the rest of the mucosal cell, almost quantitatively accounts for the entire cholesterol ester-hydrolytic activity of the whole mucosa. Table 2 also shows that, in addition to the presence of about 92% of the hydrolytic activity, nearly 33% of the synthetic activity of the whole mucosal cell was recovered in this fraction.

These results disagreed sharply with the data of Gallo & Treadwell (1963), who claimed that the brush border of rat intestine is devoid of either of the two activities. The conditions of assay employed by us and by these workers were quite different, which led us to repeat the experiments under conditions similar to those described by them. Table 2 shows that under their conditions the two enzyme activities were about equally distributed between the supernatant and brush-border fractions. The discrepancy between the results obtained under the two different sets of conditions is probably due to the different periods of incubation. The shorter period of 2–3hr. (Murthy & Ganguly, 1962) is likely to give a more correct picture of the enzyme activities than the period of 18–24hr. used by Gallo & Treadwell (1963).

It might be argued that in our experiments with the everted sacs the use of fixed pH for the respective reactions could have given such differential activities. But it is shown in Fig. 2 that on gradually changing the pH from 5·1 to 9·6 no synthesis of the sterol ester could be noticed in the medium at any pH, although the pH optimum for the cholesterol ester-synthesizing enzyme of rat intestine has been shown to be at pH 6·1 (Murthy & Ganguly, 1962). On the other hand, similar changes in the pH of the incubation medium led to very sharp changes in the efficiency of hydrolysis of the sterol ester of the medium. Strangely enough the pH optimum

Table 1. *Hydrolysis and synthesis of cholesterol esters by the mucosal homogenates and everted sacs of rat intestine*

The mucosa was removed from the weighed intestine and homogenized in 0·25M-sucrose. Everted intestinal sacs were prepared and incubated as described in the Materials and Methods section. Enzyme activities are expressed as mμmoles of cholesterol liberated or esterified/hr./g. of intestine. The results are averages of four separate experiments in each group. The figures in parentheses represent the range of each value.

Intestinal preparation	Hydrolytic activity	Synthetic activity
Mucosal homogenate	1300 (1240–1450)	2000 (1800–2200)
Everted sacs	1450 (1360–1590)	Nil

Table 2. *Distribution of the hydrolytic and synthetic activities for cholesterol esters in the brush-border and supernatant fractions of rat-intestinal mucosa*

Enzyme activities are expressed as mμmoles of cholesterol liberated or esterified/mg. of protein/hr. and are averages of six or more individual experiments. The figures under (*a*) represent the values obtained with the method of Murthy & Ganguly (1962) and those under (*b*) are the corresponding values obtained by using the procedure of Gallo & Treadwell (1963). Figures in parentheses are the ranges for each mean value.

	Hydrolytic activity		Synthetic activity	
Mucosal fraction	(*a*)	(*b*)	(*a*)	(*b*)
Brush border	83 (72–95)	37 (28–43)	48 (41–56)	36 (27–39)
Supernatant	9 (0–14)	38 (24–42)	99 (79–110)	43 (33–51)

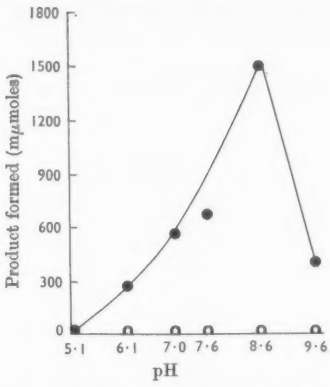

Fig. 2. Effect of pH on the hydrolysis and synthesis of cholesterol ester by the everted intestinal sacs. The conditions of the experiments are similar to those described in the Materials and Methods section. Veronal buffer (0·1 M) was used throughout and all reactions were stopped after incubation for 2 hr. Here also there was no perceptible esterification of the sterol in the medium, as can be seen from the points (○) on the base line. ●, Hydrolysis of cholesterol oleate; ○, synthesis of cholesterol oleate.

obtained with the everted sac was identical with that found for the solubilized mucosal hydrolytic enzyme (Murthy & Ganguly, 1962).

The above experiments have thus established that the brush border displays a high hydrolytic activity towards cholesterol ester, and this could lead to the assumption that this particular fraction may take an active part in the overall process of absorption of cholesterol, because this sterol is absorbed only in the unesterified form (Friedman *et al.* 1956; Treadwell *et al.* 1962). The results presented in Table 3, however, show that, apart from its enzymic potential, the brush border also plays a vital role in the actual entry of cholesterol into the mucosal cells. Thus, not only is the brush border very rich in its endogenous cholesterol content, but it also rapidly takes up the fed sterol. It can be seen from Table 3 that, compared with the rest of the cell, the brush border takes up the fed sterol earlier, and that the specific activity of the sterol in this fraction is not only higher at all periods but also reaches its peak earlier. What is most striking is the total absence of cholesterol ester from the brush-border fraction even after feeding with cholesterol, the sterol in it always being in the unesterified state. The supernatant fraction also is rich in its endogenous cholesterol content, but, in contrast with the total absence of the esterified sterol from the brush border, this fraction always contained small amounts of the sterol esters. Here also the fed labelled sterol appeared rapidly, although a little later than in the brush border. The specific activities of both the esterified and unesterified sterol in the supernatant reached their peak at about the same time and declined at about the same rate.

Further, that prior hydrolysis of the fed cholesterol ester is essential for the absorption of the sterol into the mucosal cells is demonstrated in Table 3, where it will be seen that, after feeding with [4-^{14}C]cholesterol stearate, at no time-interval were we able to detect even traces of the esterified labelled sterol in the brush-border fraction, whereas it always contained significant amounts of the unesterified labelled sterol. The supernatant fraction, however, gave a picture similar to that obtained after feeding with the unesterified labelled sterol. In these experiments the uptake of the fed esterified sterol was slower than that of the unesterified sterol; this is probably because the fed ester has to await hydrolysis before absorption.

DISCUSSION

The existence of the brush border on the columnar cells of the villi of the small intestine has been known for a long time. Yet its precise role in absorption from the intestine has not been properly understood. The classical work of Gomori (1941) had demonstrated histochemically the presence of highly active phosphatase in the brush border, but this work did not define the possible role, at the enzyme level, the brush border might play in absorption in general. More recent biophysical techniques, e.g. electron microscopy, have revealed the ultrastructure of the brush border (Dalton, 1950, 1951; Granger & Baker, 1950; Weiss, 1955*a*,*b*; Gol'din, 1956; Zetterqvist, 1956; Palay & Karlin, 1959; Millington & Finean, 1962, 1963; Sjöstrand, 1963). But these demonstrations have led to the general assumption that the main, if not the only, function of the brush border is to act as a sieve and expose enormously large surface areas, thereby facilitating very rapid absorption. However, little attempt was made, till very recently, to study the enzymes of the brush border; clearly, in the absence of such vital information the precise biochemical functions of the brush border in the overall processes of absorption can be little understood.

Serious attempts at the separation of the brush border from the intestinal mucosal cells seem to have been made only recently, as testified by the successful isolation of the brush border of hamster intestine by Miller & Crane (1961). These workers also demonstrated almost quantitative localization of invertase and maltase of the mucosal cells in

Table 3. *Distribution, during absorption, of [4-^{14}C]cholesterol in the brush-border and supernatant fractions of rat-intestinal mucosa*

Values are expressed as counts/min./mg. of cholesterol. Figures in parentheses represent the net amounts of chemically determined unesterified or esterified cholesterol. Results are averages of four separate experiments in each group in those cases where cholesterol was fed, and of two such experiments in those cases where its ester was given. In the fourth column all values are nil.

Form of sterol fed	Time (min.)	Brush border		Supernatant	
		Unesterified	Esterified	Unesterified	Esterified
Unesterified	15	1076	Nil	553	539
		(1·66)		(3·28)	(0·36)
	30	1300		706	590
		(2·24)		(2·27)	(0·33)
	90	1498		1196	655
		(2·12)		(2·88)	(0·36)
	120	1685		1295	897
		(1·90)		(2·94)	(0·11)
	150	2459		1422	1200
		(2·08)		(1·60)	(0·33)
	180	2288		1635	1918
		(2·08)		(3·54)	(0·24)
	240	2196		1521	1227
		(2·01)		(3·18)	(0·18)
	360	1397		401	1000
		(2·24)		(2·88)	(0·24)
	720	596		283	550
		(2·24)		(2·60)	(0·30)
	1440	186		205	270
		(2·40)		(2·90)	(0·13)
	2880	77		74	139
		(1·40)		(1·70)	(0·3)
Esterified (stearate)	5	90		15	Nil
		(2·74)		(3·00)	(0·25)
	10	180		36	Nil
		(1·90)		(3·00)	(0·26)
	60	320		136	Nil
		(2·01)		(3·00)	(0·24)
	120	613		368	632
		(1·61)		(2·24)	(0·24)
	180	1125		992	745
		(3·10)		(3·12)	(0·19)

this fraction. Holt & Miller (1962) demonstrated the predominance of yet two more enzymes, namely leucine amino peptidase and alkaline phosphatase in the isolated brush border of hamster mucosa. Gallo & Treadwell (1963) found about 96% of the total invertase activity of the rat-mucosal cells in the isolated brush-border fraction, although Dahlqvist & Thomson (1964) do not seem to believe that the total invertase activity of the rat-mucosal cells resides in this fraction only. Our results fully confirm the claims of Miller & Crane (1961); the values reported by Gallo & Treadwell (1963) appear to be on the high side. We therefore believe that invertase can be used as a marker for this fraction, and have followed it in this work. Although a very active alkaline phosphatase is present in the brush border (Holt & Miller, 1962), this enzyme cannot be as good a marker as invertase, because, as indicated before, during the successive stages of washing of the brush border it tends to leave the particulate sediment and appear in the supernatant.

The present work seems to be the first attempt at defining a role that the brush border might play in the absorption of cholesterol. Thus in the experiments with the everted intestinal sacs we found very rapid hydrolysis of the cholesterol ester; this suggested that the hydrolytic enzyme of the everted sacs must be in very intimate contact with the substrate. In fact, we have shown that, when separated from the rest of the mucosal cell, 92% of the total cholesterol ester-hydrolytic activity of the whole cell can be recovered in the brush-border fraction. In contrast with the very rapid hydrolysis

of the medium ester, the everted sacs showed no synthesis of cholesterol ester even up to 3hr. of incubation. Some esterification, however, did take place when incubations were carried over a very long period. This was probably due to the disruption of the cells caused by the prolonged incubation, which was accompanied by constant shaking. Such an interpretation would appear to be valid, because, although the intact cells of the everted sacs showed no esterification, homogenization of the mucosal cells revealed the presence of a highly active esterifying enzyme in the cell. But a part of the esterifying activity of the whole cell was recovered in the brush border. Therefore it appears that in the intact intestine the hydrolytic activity of the mucosa must be situated on the outer surface of the brush border and must be oriented in such a way that it constantly meets its substrate. The esterifying enzyme of the brush border, on the other hand, is probably oriented towards the inner side of the cell. Such a spatial arrangement of this enzyme would explain the inability of the everted sacs to esterify the medium sterol. These results closely resemble our earlier observations on the localization of the hydrolytic enzyme for retinyl esters on the mucosal membrane, because in these experiments also we demonstrated a similar rapid hydrolysis of the medium retinyl esters by the everted intestinal sacs of rats (Mahadevan, Seshadri Sastry & Ganguly, 1963).

We had previously shown that, after feeding with [4-^{14}C]cholesterol, the ester fraction of the labelled sterol of rat-intestinal mucosa is found distributed among the various fractions of the mucosal cell, with the microsomal fraction showing the highest activity, followed by the cell-sap fraction (Murthy *et al.* 1963). In the present experiments we have never found even traces of the ester of the labelled sterol in the isolated brush-border fraction (Table 3). It would thus be quite logical to conclude that the brush border isolated by us is free of contamination from the rest of the mucosal cell or its organelles. Therefore the activity of the synthetic enzyme as found in the isolated brush border must belong to this fraction. Such a highly active enzyme does not seem to play any role in esterifying the sterol during its absorption and it is pertinent to query whether the mere demonstration of the presence of an enzyme in any tissue must necessarily lead to a claim for its metabolic role in that particular tissue.

In our previous work, where water-soluble enzymes prepared from acetone-dried rat pancreas were used, we effected a clear separation of the hydrolytic and synthetic cholesterol esterases, and we concluded that the two activities are due to separate enzymes (Murthy & Ganguly, 1962). We have now demonstrated a markedly different distribution of the two activities in the different areas of the cell of rat-intestinal mucosa, and this was achieved by the minimum dislocation of the normal organization of the mucosal cell. Shyamala, Lossow & Chaikoff (1965) have reported a similar differential localization of the two activities in the homogenates of bovine adrenal glands.

We had earlier suggested that cholesterol is probably absorbed by a mechanism of displacement of the endogenous cholesterol of the lipoproteins that constitute the membranous materials of the mucosal cells (Ganguly, Krishnamurthy & Mahadevan, 1959). Subsequent work with labelled sterol substantiated such a hypothesis, because we demonstrated rapid and preferential appearance of the fed labelled sterol in the microsomal fraction of the mucosal-cell homogenates (Murthy *et al.* 1963). In all this earlier work we used the whole mucosal cell and did not attempt to isolate the brush border. Separation of the brush border from the rest of the cells has now enabled us to demonstrate not only that the brush border is very rich in endogenous cholesterol, but also that it rapidly takes up the fed cholesterol without effecting any quantitative changes in its cholesterol content. Therefore the theory of the absorption of cholesterol by a mechanism of displacement can be extended to the brush border also. Further, at the earlier time-intervals after the feeding of labelled sterol, not only was the specific activity of the labelled sterol in the brush border markedly higher than that in the rest of the cell, but also its peak appeared earlier in the brush border than in the rest of the cell. Even more significant is the total absence of any cholesterol ester in the brush-border fraction despite the presence of abundant amounts of the substrate and a very active esterifying enzyme in the isolated brush border. Moreover, the total absence of esterified sterol from the brush-border fraction, even after feeding of the sterol ester, confirms the observations of the earlier workers that the cholesterol esters have to be totally de-esterified before the sterol can be absorbed (Friedman *et al.* 1956; Treadwell *et al.* 1962); this de-esterification seems to be a significant rate-limiting step, because, at all time-intervals after feeding of comparable amounts of the labelled sterol in the esterified or unesterified state, markedly higher radioactivity could consistently be located in all the fractions of the mucosal cell of those rats that received the unesterified sterol. The brush border thus appears to play at least two major roles in the absorption of cholesterol: it can complete the hydrolysis of the fed sterol esters, which under normal circumstances is probably initiated by the more powerful pancreatic enzyme; this is then followed by the rapid uptake of the de-esterified sterol by the brush border.

Another important event in the process of absorption of cholesterol is its re-esterification, because the absorbed sterol eventually appears in the lymph mostly as its ester (Swell *et al.* 1959; Treadwell *et al.* 1959). It has already been discussed that no sterol ester can be detected in the isolated brush border after feeding with esterified or unesterified cholesterol. On feeding the labelled unesterified sterol the specific activity of the cholesterol ester in the supernatant steadily increased, reaching a peak at 180 min., after which it declined, and at all time-intervals the net amount of cholesterol ester in this mucosal fraction remained fairly constant (Table 3). These results show that re-esterification takes place inside the mucosal cell and that the rate of re-esterification is parallel to the rate of removal of the ester from the mucosal cell. On the basis of the results of the present work it is therefore possible to visualize the following sequence of events in the absorption of cholesterol: the dietary esters of cholesterol are hydrolysed by the cholesterol ester hydrolase of either the pancreas or the mucosal brush border, or by both, after which the de-esterified sterol is rapidly taken up by the brush border and transferred into the mucosal cell, where it is slowly re-esterified for transport through lymph.

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The Dietary-Regulated Biosynthesis of High-Sulphur Wool Proteins

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1. When the diet of sheep is supplemented by the abomasal infusion of sulphur-containing amino acids or casein, a special group of proteins, with a very high content of sulphur (about 8·3%), is incorporated into the high-sulphur proteins of wool. These special proteins cannot be detected in control wool from the same sheep. 2. This is a naturally occurring process, as these special proteins are found in wool from sheep on a high level of nutrition under ordinary conditions of feeding, and in wool of an inherently high sulphur content. 3. This represents a control mechanism in protein synthesis that has not previously been observed, and may be further evidence that the high-sulphur proteins of wool are produced by an unusual synthetic route.

Reis & Schinckel (1963, 1964) showed that the rate of wool growth was substantially increased by infusing cysteine, methionine or casein directly into the abomasum of sheep. The sulphur content of wool was increased by as much as 35% as a result of such abomasal feeding. The observation that the sulphur content of wool is also altered substantially by changes in the nutritive status of sheep in controlled feeding experiments (Reis, 1965*a*,*b*) or under free-grazing conditions (Reis & Williams, 1965) implies that the mechanism for increased sulphur incorporation is a normal attribute of the animal and not a special property induced by the technique of abomasal feeding.

Wool keratins are complexes of two main types of protein: those with sulphur contents less than that of the parent wool (low-sulphur proteins) and a second fraction made up of a mixture of many proteins of sulphur content higher than that of the whole wool (high-sulphur proteins). An investigation of sulphur-enriched wool samples grown during abomasal feeding (Gillespie, Reis & Schinckel, 1964) showed that the increase in sulphur content was restricted to the high-sulphur proteins of the wool fibre. The proportion of the high-sulphur proteins was increased, and within this group of proteins there was increased formation of those protein components richest in sulphur. Within experimental error, these changes accounted for the increased sulphur content of the wool.

Electrophoresis by the moving-boundary technique at pH 4·5 and in starch gel at pH 8·0 gave no evidence of new protein components in the sulphur-enriched wool samples and suggested that there had been an increased synthesis of the same proteins present in control wool. However, this was not entirely compatible with the evidence from amino acid analysis, which showed that the high-sulphur proteins from sulphur-enriched wool contained very much less aspartic acid, phenylalanine and leucine than did those from control wool. These differences could possibly be due to the production of the same protein components in both control and sulphur-enriched wool, but in different proportions, or more likely to the production of protein components that are not present in detectable amounts in control wool.

Further evidence is presented below which suggests that the second alternative is correct and that a special group of protein components is in fact formed during sulphur-enrichment. It is further suggested that this is a naturally occurring process as these special proteins are found in wool from sheep on a high level of nutrition and in wool of an inherently high sulphur content.

EXPERIMENTAL

Experimental procedures with sheep. Most sheep were kept indoors and were fed individually. The animals studied were English Leicester, Merino and English Leicester × Merino crosses. In some experiments the nutrition was varied by altering the amount or composition of the diet (Reis, 1965*a*,*b*), whereas in other experiments a basal diet

of equal parts of lucerne chaff and wheaten chaff was supplemented with sulphur-containing amino acids or casein, infused directly into the abomasum (Reis & Schinckel, 1963, 1964). Wool was collected at regular intervals, usually 3 weeks, from areas (approx. 10cm. × 10cm.) that were defined by tattooing. Control samples were obtained from these areas on each animal before the diet was altered

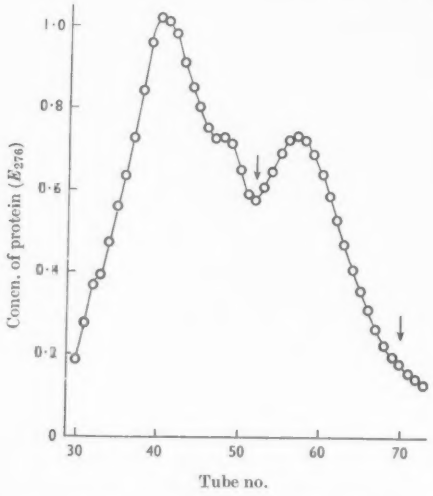

Fig. 1. Chromatography of high-sulphur proteins on DEAE-cellulose at pH 4·5. Experimental details are given in the text.

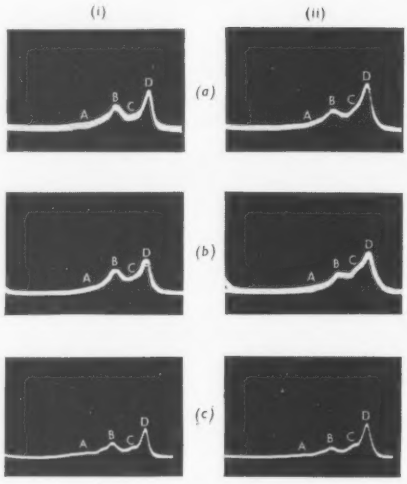

Fig. 2. Moving-boundary electrophoretic patterns of high-sulphur proteins from runs at pH 4·5 showing the differences between the proteins from control (i) and sulphur-enriched wools (ii) grown by three different sheep. (*a*) Merino (sheep SC 8): control wool, 2·84% of S; sulphur-enriched wool, 3·72% of S; abomasal infusion of 60g. of gelatin and 3·7g. of DL-methionine/day. (*b*) English Leicester (sheep 3307): control wool, 3·04% of S; sulphur-enriched wool, 3·96% of S; abomasal infusion of 3·7g. of DL-methionine/day. (*c*) English Leicester × Merino (sheep 1391): control wool, 3·06% of S; sulphur-enriched wool 3·88% of S; abomasal infusion of 60g. of gelatin and 3·0g. of L-cysteine/day.

Table 1. *Effect on wool composition of the abomasal infusion into sheep of cysteine and methionine, alone and in combination with gelatin or casein*

Sheep	Breed	Wool sample no.	Abomasal supplement	Sulphur content (%)	Content of high-sulphur protein (%)	Sulphur content of high-sulphur protein (%)
1390	English Leicester × Merino	63	None	3·04	22	4·9
		65	Casein and cysteine	3·98	31	5·7
1391	English Leicester × Merino	66	None	3·06	22	4·9
		68	Gelatin* and cysteine	3·88	30	6·5
SD67	English Leicester × Merino	331	None	3·08	21	4·8
		332	Methionine	4·17	32	6·1
3307	English Leicester	333	None	3·04	24	4·8
		334	Methionine	3·96	33	6·0
SC8	Merino	136	None	2·84	20	4·8
		137	Gelatin* and methionine	3·72	29	5·8

* Gelatin alone had no effect on the growth of wool or its composition.

or supplemented via the abomasum. Wool samples were also obtained from sheep in an experimental flock in the field by clipping at intervals of 6 weeks.

Preparation of wool samples. Each sample of wool was washed thoroughly in light petroleum (b.p. 30–80°), in ethanol and in water and was finally equilibrated in a conditioned room (20°; 60% relative humidity).

Sulphur analysis. The sulphur content of wool was measured by an oxygen flask technique as described by Reis & Schinckel (1963).

Isolation and estimation of wool proteins. Wool samples were extracted with a solution at pH11·0 of 8M-urea containing potassium mercaptoacetate (0·2M) for 2hr. at 40°. The extracted proteins, which represented about 90% of the fibre, were converted into *S*-carboxymethylkerateines by alkylation with iodoacetate and then dialysed. After removal of the low-sulphur fraction by precipitation with zinc acetate at pH6·0 (final concn. 20mM), the high-sulphur proteins in the supernatant were recovered by dialysis and freeze-drying. Complete details of the extractions and of the procedures used to measure the proportions of proteins in the extracts have been described by Harrap & Gillespie (1963) and Gillespie (1964).

Chromatographic separation of high-sulphur protein components. Freeze-dried protein (1g.) dissolved in 60ml. of acetic acid–sodium acetate buffer, pH4·5 and *I* 0·02, was applied to a column (5cm.×15cm.) of DEAE-cellulose (EK7392) and eluted at room temperature with a linear gradient of 0·25–0·8M-NaCl in acetate buffer. The total volume of eluent was 1500ml. and 15ml. fractions were collected. The protein concentration of the column effluent was continuously monitored by measuring the extinction at 278mμ with an instrument designed for the purpose by Gilson Medical Electronics. Fig. 1 shows a typical elution profile. This degree of resolution was adequate for the present purpose; however, it can be increased by using a shallower gradient and a lower load of protein (Gillespie, 1963*a*,*b*). Protein components running in electrophoretic peak D (see Fig. 2) were isolated by pooling the fractions eluted between the points indicated by arrows on Fig. 1, dialysing and freeze-drying. As some variation occurred in the position of eluted protein peaks from different protein preparations, the elution profile was plotted in each experiment and the position of the minimum used as the reference from which fractions were collected.

Moving-boundary electrophoresis. The proportions and mobility of high-sulphur protein fractions were estimated by electrophoresis in a Tiselius moving-boundary apparatus (LKB Produktor, Stockholm, Sweden). Freeze-dried proteins were dissolved in the appropriate buffer to give a 1·1–1·2% (w/v) solution and then dialysed for 24hr. against 2l. of the same buffer. The following buffers of ionic strength 0·1 were used; acetic acid–sodium acetate buffer, pH4·5; veronal buffer, pH8·6; glycine–NaOH buffer, pH10·0; β-alanine–NaOH buffer, pH11·0. The experiments were continued until the fastest ascending peak traversed the full length of a standard long intermediate cell. The proportions of components in the proteins were estimated from the relative areas under peaks in the ascending patterns. On tracings, the peaks were arbitrarily defined by vertical lines, the horizontal distance covered under each peak being kept the same in control and sulphur-enriched wool patterns to make the results strictly comparable.

Amino acid analysis. Samples of protein (30mg.) were hydrolysed *in vacuo* at 108° for 24hr. with 5ml. of 6N-HCl. The HCl was removed by freeze-drying and the content of amino acids in the hydrolysate estimated by ion-exchange chromatography with an automatic amino acid analyser (Beckman–Spinco).

Table 2. *Amino acid composition of high-sulphur proteins isolated from control and sulphur-enriched wool produced by Merino × English Leicester sheep* 1390

The results are expressed as amino acid N as percentage of total N. Where sulphur-enrichment has resulted in a change in amino acid composition exceeding 10% this has been indicated.

	High-sulphur protein			Peak D fraction		
Amino acid	Control	Sulphur-enriched	Difference (%)	Control	Sulphur-enriched	Difference (%)
Lysine	1·05	0·96		0·50	0·62	
Histidine	1·58	1·66		0·64	1·05	
Arginine	12·10	12·56		18·27	18·17	
S-Carboxymethylcysteine	14·37	16·16	+12	16·49	18·76	+14
Aspartic acid	2·43	1·94	−20	1·08	0·77	−29
Threonine	7·77	8·02		7·44	7·62	
Serine	9·39	9·79		10·20	10·50	
Glutamic acid	6·01	6·26		6·74	6·48	
Proline	9·42	9·96		9·18	9·23	
Glycine	5·23	4·61	−12	5·11	4·54	−11
Alanine	2·52	2·03	−19	2·40	1·92	−20
Valine	4·61	4·17		3·71	3·40	
Isoleucine	2·39	2·22		2·25	2·09	
Leucine	3·11	2·36	−24	1·98	1·53	−23
Tyrosine	0·59	0·42		1·68	1·62	
Phenylalanine	1·54	0·96	−37	0·86	0·65	−24

RESULTS

Protein composition of sulphur-enriched wool. Supplements of DL-methionine or L-cysteine, either alone or in combination with gelatin or casein, infused directly into the abomasum substantially increased the sulphur content of wool (Table 1). This was accompanied by an increase in the proportion of high-sulphur protein and an increase in the sulphur content of that fraction. Although the accurate estimation of the proportion of high-sulphur proteins is difficult, the evidence suggests that these changes in the high-sulphur proteins account for the increased sulphur content of the wool. Thus an examination of the electrophoretic patterns (Fig. 2) and the data in Table 1 indicate that the increase in the high-sulphur protein content of sulphur-enriched wools is entirely due to the increased synthesis of protein with mobilities in the ranges of peaks C and D. This conclusion applies irrespective of the breeding of the sheep (Merino, English Leicester or English Leicester × Merino). The present investigation is confined to an examination of the changes in the proteins within peak D: first, because changes in this region account for about 60% of the total change, and, secondly, because at present peak D proteins are more readily separated from the mixture by column chromatography.

Amino acid composition of high-sulphur proteins isolated from control and sulphur-enriched wool. Comparative amino acid analyses of high-sulphur proteins originating from control and sulphur-enriched wools are shown in Table 2. Besides showing the expected increase in *S*-carboxymethylcysteine content due to sulphur-enrichment, the unfractionated protein also shows large decreases in aspartic acid, glycine, alanine, leucine and phenylalanine. Similar changes occur in the composition of peak D protein as a result of sulphur-enrichment. These results suggest the synthesis of a protein high in cystine and low in aspartic acid, glycine, alanine, leucine and phenylalanine but in other respects similar in composition to high-sulphur proteins from control wool. It was decided to investigate whether this is a new synthesis of a novel protein or merely increased synthesis of a component present in control wool.

Detection of new protein components. A search was made for new protein components by varying the conditions of electrophoresis. In terms of the number of visible components obtained, resolution of unfractionated high-sulphur proteins steadily worsens as the pH of electrophoresis is varied away from 4·5 (Gillespie, 1963*a*,*b*). However, on electrophoresis of sulphur-enriched high-sulphur protein in the pH range 10–11, there was evidence of a fast component not present, or present to only a small extent, in the control high-sulphur protein (Fig. 3). To identify this material, peak D proteins were isolated chromatographically and run at pH11·0; a clear difference was seen between the patterns of the fractions from control and sulphur-enriched wool samples. Peak D protein from a control wool (sheep 1390) showed essentially only a single boundary at pH4·5, 8·6, 10·0 and 11·0, whereas peak D protein from sulphur-enriched wool from this sheep separated into two peaks at pH11·0 (D1 and D2; Fig. 4). The resolution at pH10·0 was not quite as good as at pH11·0 but, as there was some evidence of protein degradation on continued

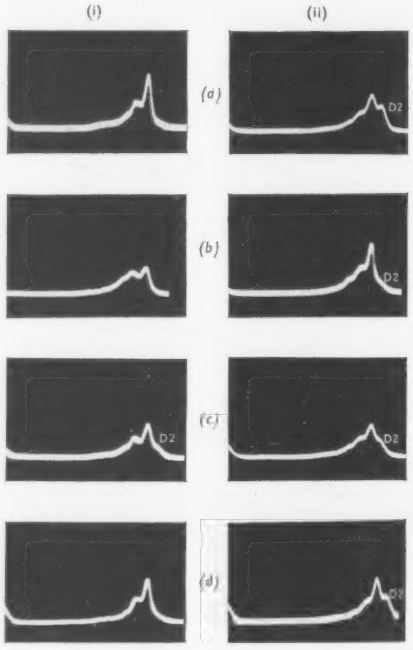

Fig. 3. Moving-boundary electrophoretic patterns of high-sulphur proteins run at pH11·0 showing the differences between control (i) and sulphur-enriched (ii) wools grown by a number of English Leicester × Merino sheep. (*a*) Sheep 1391: control wool, 3·06% of S; sulphur-enriched wool, 3·88% of S; abomasal infusion of 60g. of gelatin and 3·0g. of L-cysteine/day. (*b*) Sheep 1392: control wool, 3·15% of S; sulphur-enriched wool, 3·73% of S; abomasal infusion of 60g. of casein and 1·5g. of L-cysteine/day. (*c*) Sheep 1393: control wool, 3·38% of S; sulphur-enriched wool, 3·95% of S; abomasal infusion of 60g. of gelatin and 1·5g. of L-cysteine/day. (*d*) Sheep SD67: control wool, 3·08% of S; sulphur-enriched wool, 4·17% of S; abomasal infusion of 3·7g. of DL-methionine/day.

dialysis at pH11·0, electrophoresis at pH10·0 was employed in some experiments.

Peak D2 has been identified as the new peak in two ways. (1) Peak D fractions were isolated from wool samples enriched with sulphur to only a moderate degree and run electrophoretically at pH11·0. Peak D2 was still observable but was present in diminished amount as compared with the fractions from fully enriched wool (Fig. 5). It is clear that the proportion of peak D2 increases with the degree of sulphur-enrichment of the wool. (2) When a mixture of equal parts of peak D protein from control and sulphur-enriched wool samples (sheep 1391) was run electrophoretically at pH11·0, the area under peak D2 (Fig. 6) decreased by about the expected 50%.

There seem to be ample grounds for identifying peak D2 with the fastest peak observed in runs at pH11·0 with unfractionated high-sulphur protein from sulphur-enriched wool (Fig. 3). It has therefore been possible to dispense with the preliminary fractionation and run the unfractionated protein at pH11·0 to assess the proportion of peak D2 material.

Properties of peak D2 protein. The proteins of peak D2 have not yet been completely separated from peak D1 proteins although a purity of about 90% has been obtained by fractional precipitation and chromatography. Even though this fraction is not completely pure the amino acid analysis is noteworthy (Table 3), for compared with other high-sulphur proteins this fraction has a strikingly high content of *S*-carboxymethylcysteine, a very low content of aspartic acid, leucine and phenylalanine and a comparatively low content of glycine, alanine and valine. This composition is extremely close to that which would be predicted from the observed differences in amino acid composition of the high-sulphur proteins and fractions isolated from control and sulphur-enriched wool (Table 3).

Differences between breeds of sheep. The proteins discussed so far have been extracted from the wool of English Leicester × Merino sheep. High-sulphur proteins have also been obtained from control and sulphur-enriched wool grown by Merino and English Leicester sheep and in each instance the proportion of protein in peaks C and D greatly increased during sulphur-enrichment (Fig. 2). Calculations showed that, as in the experiments above, changes in the high-sulphur proteins accounted for substantially all the increases in sulphur content of the wool. When peak D fractions from these wools were run

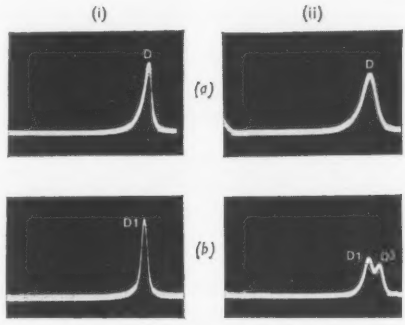

Fig. 4. Moving-boundary electrophoretic patterns of peak D fraction of high-sulphur proteins isolated from control (i) and sulphur-enriched (ii) wool produced by an English Leicester × Merino sheep (1390). Control wool, 3·04% of S; sulphur-enriched wool, 3·98% of S; abomasal supplement of 60g. of casein and 3·0g. of L-cysteine/day. (*a*) Electrophoresis at pH4·5; (*b*) electrophoresis at pH11·0.

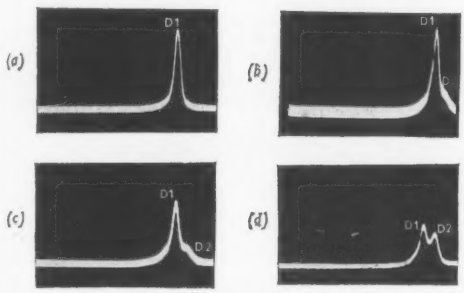

Fig. 5. Moving-boundary electrophoretic patterns of peak D high-sulphur proteins run at pH11·0, showing the increase in proportion of D2 protein with increasing degree of sulphur-enrichment of wool. (*a*) Wool: 3·04% of S; no detectable peak D2; control wool. (*b*) Wool: 3·48% of S; 18% of peak D2; abomasal infusion with 60g. of casein/day. (*c*) Wool: 3·60% of S; 21% of peak D2; abomasal infusion with 60g. of casein/day. (*d*) Wool: 3·98% of S; 39% of peak D2; abomasal infusion with 60g. of casein and 3·0g. of L-cysteine/day. Samples (*a*), (*c*) and (*d*) were from English Leicester × Merino sheep 1390; sample (*b*) was from English Leicester × Merino sheep 1392.

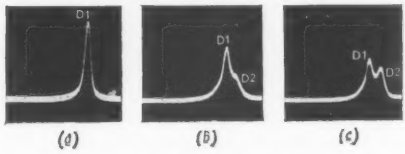

Fig. 6. Moving-boundary electrophoretic patterns of peak D high-sulphur proteins isolated from (*a*) control, (*c*) sulphur-enriched wools (sheep 1390) and (*b*) a mixture (1:1) of these two fractions. Runs were made at pH11·0.

Table 3. *Amino acid composition of partially purified peak D2 fraction of high-sulphur protein*

The results are expressed as amino acid N as percentage of total N. For comparison, predicted compositions are given; these have been calculated from the differences in amino acid composition between high-sulphur protein and fractions isolated from control and sulphur-enriched wool samples.

		Predicted composition of peak D2 proteins	
Amino acid	Amino acid composition of impure peak D2 protein	From differences in composition between high-sulphur protein isolated from six pairs of control and sulphur-enriched wool samples	From difference in composition between peak D proteins isolated from control and sulphur-enriched wool, assuming that peaks D1 and D2 contain equal amounts of protein (sheep 1390)
Lysine	1·23	—	0·74
Histidine	2·27	—	1·46
Arginine	16·1	—	18·1
S-Carboxymethylcysteine	22·1	21·2	21·1
Aspartic acid	0·49	0·5	0·46
Threonine	8·39	—	7·72
Serine	9·56	—	10·8
Glutamic acid	6·69	—	6·31
Proline	8·96	—	9·25
Glycine	3·01	3·1	3·97
Alanine	1·99	1·4	1·44
Valine	3·08	3·2	3·09
Isoleucine	1·27	1·6	2·00
Leucine	1·12	0·8	1·08
Tyrosine	1·10	—	1·60
Phenylalanine	0·22	0·2	0·44

at pH11·0 (Fig. 7) in each case peaks D1 and D2 were resolved, but the ratio of peak D2 to D1 was lower than found in the wool proteins from most of the cross-bred sheep (Figs. 3 and 7).

Presence of peak D2 proteins in wool from normally fed sheep. (*a*) Wool grown during periods of high nutritive status. In experiments with pen-fed sheep, Reis (1965*a*,*b*) found that increasing the level of nutrition of sheep increased the sulphur content of the wool they produced. It has been shown that, as found with abomasal supplements, this increase in sulphur content of the wool is caused by an increase in the level of high-sulphur protein in the wool, and by an increase in the proportion of the proteins in peaks C and D (Gillespie, 1965; and Fig. 8*a*). Electrophoresis at pH10·0 (Fig. 8*b*) shows that this increase in the sulphur content of wool, caused by high nutritional status, is paralleled by an increase in the proportion of peak D2 protein.

(*b*) Wool of different sulphur content produced by sheep on equal levels of nutrition. Wool samples were obtained from two sheep pen-fed at the same level of nutrition (Reis, 1965*a*,*b*). One sheep (E 113; a high-producer of wool) grew wool of a low sulphur content (3·00%), whereas the other (E 123; a low-producer of wool) provided wool of comparatively high sulphur content (3·65%). It was found that the latter wool contained more high-sulphur protein (25% compared with 20%) and specifically more of the proteins in peaks C and D than did the wool of lower sulphur content (Fig. 9*a*). Electrophoretic analysis at pH 11·0 of peak D protein isolated from these wool samples (Fig. 9*b*) showed that wool from sheep E 113 contained almost none of peak D2 protein whereas wool from sheep E123 contained a substantial amount.

(*c*) Seasonal variation in sulphur content of wool from grazing sheep. Wool samples, clipped from grazing animals at 6-weekly intervals throughout a year, were analysed for sulphur content. Wool from the same animal showed considerable variations in this respect, the range for an individual animal being from 3·06 to 3·74%. These seasonal variations are most probably due to variable nutrition (Reis & Williams, 1965). High-sulphur proteins were isolated from wool samples at the

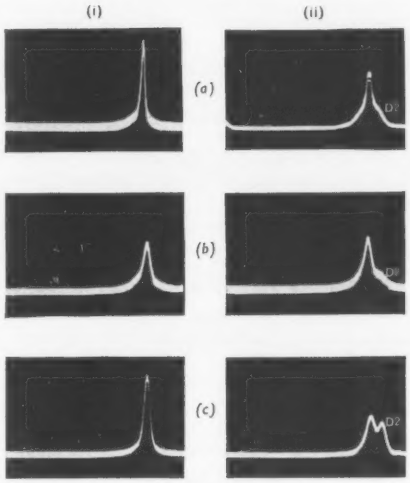

Fig. 7. Moving-boundary electrophoretic patterns of peak D high-sulphur proteins run at pH11·0 showing the differences between control (i) and sulphur-enriched (ii) wools produced by (*a*) Merino sheep SC8, (*b*) English Leicester sheep 3307 and (*c*) English Leicester × Merino sheep 1390. The abomasal supplements provided and the sulphur contents of the wools are given with Figs. 2 and 4.

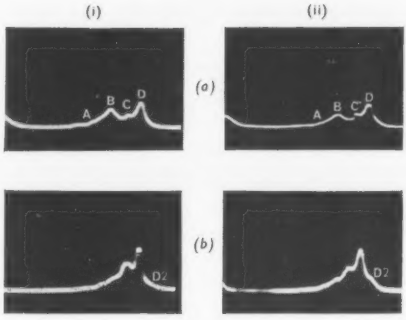

Fig. 8. Moving-boundary electrophoretic patterns of high-sulphur proteins isolated from wools of different sulphur contents produced by Merino sheep E109 under different levels of nutrition: (i) low-nutrition wool (3·04% of S); (ii) high-nutrition wool (3·65% of S). (*a*) Electrophoresis at pH4·5; (*b*) electrophoresis at pH10·0.

extremes of the ranges and were run electrophoretically at pH4·5 and 10·0 (Fig. 10): the increase in sulphur content in wool is paralleled by an increase

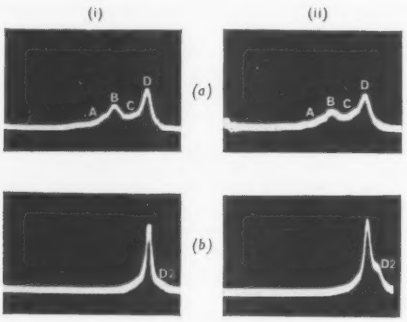

Fig. 9. Moving-boundary electrophoretic patterns of high-sulphur proteins isolated from wools of different sulphur content produced by two Merino sheep receiving the same level of nutrition. The sheep had equal food intakes but differed in having a high production of wool of 3·00% S content (sheep E113) (i) and a low production of wool of 3·65% S content (sheep E123) (ii). (*a*) Electrophoresis at pH4·5; (*b*) electrophoresis of peak D fraction at pH11·0.

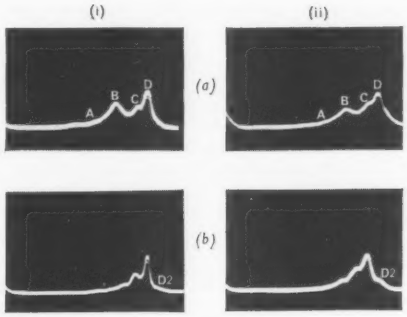

Fig. 10. Moving-boundary electrophoretic patterns of high-sulphur proteins isolated from wool samples of different sulphur content clipped from Merino sheep 773 at different times during the year: (i) wool of 3·06% S content; (ii) wool of 3·74% S content. (*a*) Electrophoresis at pH4·5; (*b*) electrophoresis at pH10·0.

in the proportion of peaks C and D and by an increased proportion of peak D2 protein.

DISCUSSION

As the result of this and previous work (Gillespie *et al.* 1964) it is now well established that the increased sulphur content of wool produced during

the abomasal infusion of sulphur-containing amino acids into sheep can be accounted for by an increased synthesis of those high-sulphur proteins that fall within the mobility ranges of peaks C and D. In most of the English Leicester × Merino sheep used in the present study the increased synthesis of peak D protein, accounting for about 60–70% of the total, is largely confined to the formation of the proteins of peak D2 that are not present, or present to only a limited extent, in the controls.

The protein material in peak D2 differs from the other high-sulphur proteins by its higher content of *S*-carboxymethylcysteine, its lower content of glycine, alanine and leucine and its extremely low content of phenylalanine and aspartic acid. The presence of peak D2 proteins in sulphur-enriched wool, produced during abomasal infusions, is sufficient to account for most of the changes that take place in the amino acid composition of the high-sulphur proteins (Table 3). That these proteins are not produced by an abnormal process is shown by their occurrence in wool of high-sulphur content produced by sheep on normal diets, both indoors and in the field. The nature of the changes in the proteins in peak C has not yet been examined.

Less peak D2 protein was produced by English Leicester (3307) and Merino (SC8) sheep than might be expected from the extent of sulphur-enrichment of the wool, although electrophoresis at pH4·5 showed the expected proportional increases in the proteins of peaks C and D. Although the reason for this is unknown, two explanations seem feasible. During sulphur-enrichment these sheep may produce mostly additional normal peak C and D proteins; it is also possible that peak D1 and D2 proteins are produced, but with mobilities so close that they cannot be adequately resolved by electrophoresis at pH11·0. The different response of these two sheep is probably a reflection of individual differences rather than differences between breeds. Thus Merino sheep E123 (Fig. 9*b*), although producing wool enriched to only a moderate degree, produced as much D2 fraction as did an English Leicester × Merino sheep (1390) whose wool was enriched to a similar extent (Fig. 5*c*). However, peak D2 proteins are always produced during the process of sulphur-enrichment, regardless of the type of abomasal supplement given (methionine, cysteine or casein) or the breed of sheep, although there may be individual differences in the magnitude of the response.

In the three experiments involving normally fed sheep, the higher the sulphur content of the wool, the greater was the amount of peak D2 protein it contained. It is thus reasonable to suppose that this is a generally occurring phenomenon. To what extent changes in this protein fraction quantitatively account for the normally occurring variations in the sulphur content of wool cannot yet be answered.

It has been shown above that when Merino sheep of different wool-producing capacity were provided with near identical food intake and hence of sulphur-containing amino acid intake, the faster-growing wool contained much less sulphur and peak D2 protein than the slow-growing wool. A possible hypothesis to explain this would be that there is a lower limit to the sulphur content of wool provided by a basic structure of microfibrillar protein plus a minimal amount (about 20%) of high-sulphur protein. The balance between the rate of wool growth and the amount of cysteine available to the follicles will determine the amount of sulphur in excess of the minimum required for this essential wool structure. It is probable that this excess of sulphur can be incorporated into wool as additional high-sulphur components, and of these the proteins of peak D2 provide a major fraction. We thus have evidence for the existence of an unusual biological control mechanism that may play a part in the formation of α-keratins generally.

The follicle presumably has all the necessary synthetic mechanisms for making D2 proteins but these do not function, at least to a detectable extent, until the sulphur-containing amino acid concentration reaches a particular value. It is not possible at present to define this concentration or the site where it is measured. However, this is an unusual control mechanism in protein synthesis and appears to be of a type not observed before. The control may be exercised either at the follicle or elsewhere in the body, perhaps on the formation of a hormone. If the site of action is at the follicle level, an inductive process or other direct control on the protein-synthetic mechanism may be involved. Alternatively, two not mutually exclusive ways in which mass action effects could occur can be visualized: (1) It is thought that cysteine penetrates the suprabulbar region of the follicle and then diffuses distally (Downes, Lyne & Clarke, 1962). A concentration gradient of cysteine would thus be set up, the slope of which would depend on the level of cysteine uptake by the follicle and the rate of metabolism. The proteins of peak D2 are probably among the last to be synthesized as the fibre moves along the follicle (Downes, Ferguson, Gillespie & Harrap, 1966). It is reasonable to suppose therefore that, at low levels of cysteine uptake or at high rates of metabolism, only traces of cysteine may reach this synthetic region. (2) If, as has been suggested (Gillespie, 1963*b*, 1965), high-sulphur proteins from keratins are synthesized by the stepwise addition of sulphur-rich peptides to precursors, then it might be expected that under conditions of abundant supply of cysteine this addition reaction would be pushed through to

produce the very sulphur-rich components of the D2 fraction.

We thank Miss C. M. Tomlinson and Mr D. A. Tunks for their skilled technical assistance.

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The Structure and Deposition of Human-Liver Glycogens

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1. Glycogen was extracted from and the amount determined in human foetal livers ranging in age from 13½ to 26 weeks. 2. The detailed structures of human foetal- and child-liver glycogens were examined and shown to be essentially the same. 3. The deposition of glycogen in different mammalian species is discussed.

Most studies on glycogen deposition in the foetal liver have been made on laboratory animals, e.g. rats (Jacquot, 1959), guinea pigs (Ducommun-Lehmann, 1951; Nemeth, 1954), rabbits (Jost & Jacquot, 1955) and lambs and monkeys (Shelley, 1960). In all cases the initial concentrations of the polysaccharide are very low and they tend to rise sharply in the later stages of gestation, until at birth they have reached or surpassed the normal adult level. Such studies as have been made with human foetal material (e.g. Kitamura, 1942; Shapovalov, 1961, 1962) employed histochemical techniques and indicated that a similar pattern of development occurs. Histochemical techniques should, however, be accepted with caution, as they may detect polysaccharides other than glycogens, and there is controversy about the first appearance of glycogen in the liver tissues. No determinations of the detailed structures, e.g. measurements of CL(av.)† and ECL(av.), of normal human foetal glycogens have previously been made. Data on the structures of other mammalian foetal glycogens are also limited. Chain lengths of 11 D-glucose units for a pig-liver glycogen (Manners & Archibald, 1957), 13 units for a sheep-liver glycogen (Bell & Manners, 1952) and 11·5 units for a guinea-pig-liver glycogen (Illingworth, Cori & Larner, 1952) have been reported, but no indications of the foetal ages were given. The most comprehensive survey is that by Warren & Whittaker (1959), who studied the structure of glycogens from foetal goat livers and obtained CL(av.) and ECL(av.) values in the normal range, i.e. 10–14 and 7–9 D-glucose units respectively.

The present study was undertaken in an attempt to determine the earliest stages at which recognizable glycogens may be isolated from the human foetal liver, and to examine the detailed structures of these polysaccharides and compare them with glycogens from more mature liver tissues. A preliminary account of this work has been given (Bourne, McLean & Pridham, 1964).

METHODS AND MATERIALS

Human liver specimens. These specimens, both foetal and mature, were kindly provided by Dr B. Clayton, The Hospital for Sick Children, Great Ormond Street, London, W.C. 1. These were cooled to −20° as rapidly as possible after removal from the body, and stored at this temperature.

β-Amylase. This was obtained from the Wallerstein Co., Mariners Harbor, Staten Island, N.Y., U.S.A. The units of activity are expressed as mg. of maltose liberated by 1 mg. of enzyme under the conditions specified by Hobson, Whelan & Peat (1950) but at a temperature of 30°.

α-Amylase. This was prepared by the method of Fischer & Stein (1954). The crystallization stage was omitted, and the enzyme freeze-dried in 0·2M-sodium citrate buffer, pH 7·0 (Liddle & Manners, 1957). The unit of activity is the quantity of enzyme that liberates 1 mg. of apparent maltose from 1 ml. of starch solution (1%, w/v) in 3 min. at 35° (Manners & Wright, 1962).

Glycogen extraction. Homogenization with cold aq. 10% (w/v) trichloroacetic acid solution was employed as the general method (Bloom, Lewis, Schumpert & Shen, 1950). The glycogen was then precipitated from the extract with ethanol and centrifuged, and the polysaccharide was washed with ethanol followed by ether and finally dried under reduced pressure in the presence of P_2O_5.

Determination of purity of glycogens. Glycogens were hydrolysed with 2N-H_2SO_4 at 100° for 2 hr. After appropriate dilution with water, portions were assayed for glucose by the method of Somogyi (1952).

Determination of CL(av.). Samples were analysed by incubation with α-amylase as described by Manners & Wright (1962), except that the incubation time was 15 hr.; 4 units of α-amylase/mg. of glycogen were used. Apparent

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† Abbreviations: CL(av.), average chain length; ECL(av.), average exterior chain length; ICL(av.), average interior chain length, i.e. CL(av.) − ECL(av.) − 1.

maltose production was measured by the method of Park & Johnson (1949). Larger samples were assayed by periodate oxidation (Potter & Hassid, 1948) with the modification due to Manners & Archibald (1957).

Determination of ECL(av.). When relatively large amounts of glycogen were available, a 30mg. specimen was incubated with β-amylase (1800 units) and 0·2M-sodium acetate buffer, pH4·6 (5ml.), made up to 25ml. with water, at 30° for 24hr. (Bell & Manners, 1952). The maltose liberated was determined by the method of Somogyi (1952).

For the smaller samples the digests were reduced to glycogen (1mg.), β-amylase (50–70 units) and 0·2M-sodium acetate buffer, pH4·6 (0·4ml.), in a total volume of 10ml. After suitable dilution, the maltose was measured by the method of Park & Johnson (1949).

Measurement of absorption spectra of glycogen–iodine complexes. Glycogen (2·5mg.) in iodine solution (5ml. of 0·1% I_2 in 1% KI) was made up to 25ml. with water. The absorption spectrum was measured in the range 400–700mμ in a Unicam SP.500 spectrophotometer with 1cm. glass cells. The reference solution used was 0·02% I_2 in aq. 0·2% KI.

Partial acid hydrolysis. Partial hydrolysis of glycogen was carried out by using the conditions of Wolfrom, Lassettre & O'Neill (1951). The neutralized hydrolysates were examined on paper chromatograms with ethyl acetate–acetic acid–water (9:2:2, by vol.) as solvent, and the sugars located with *p*-anisidine hydrochloride (Hough, Jones & Wadman, 1950). The hydrolysates, after reduction with $NaBH_4$, were also examined by paper electrophoresis with 0·062M-sodium molybdate solution as electrolyte (Bourne, Hutson & Weigel, 1961).

RESULTS AND DISCUSSION

Foetal livers ranging in age from 11½ to 26 weeks were examined. In most cases, liver tissues of the same foetal age were pooled to obtain sufficient material for analysis. Foetal livers aged 11½ and 12 weeks gave only traces of precipitate when extracted with trichloroacetic acid and then treated with ethanol. Partial acid hydrolysis of these precipitates, followed by chromatography and electrophoresis, revealed no reducing sugars, whereas the treatment of glycogen from a 26-week human foetal liver yielded glucose, maltose and isomaltose, thus revealing the presence of the expected α-(1→4)- and α-(1→6)-glucosidic linkages in the polysaccharide. The nature of the precipitates from the early foetal livers was not established.

Milligram quantities of glycogen were obtained from foetal livers at 13½ weeks (Table 1) and a sharp increase in the amount deposited was observed in 25–26-week livers.

The chemical and enzymic methods employed in this study demonstrated that the detailed structures of these glycogens and those from the livers of children aged from 6 weeks to 11 years (Table 2) were essentially the same. Most of the foetal glycogens (Table 1) were within the normal range of variation (see Manners, 1957). The values for CL(av.) varied from 12·5 to 14·5 D-glucose units (average 13·7) and those for ECL(av.) from 7·7 to 10·2 units (average 8·8). An absorption maximum of 470mμ for an iodine complex with the 26-week foetal glycogen fell within the normal range 420–490mμ (Manners, 1957), as did those for the other glycogen samples examined in this way (Table 3). This provided additional evidence that the foetal glycogen had a normal structure.

The CL(av.) values for the child-liver glycogens fell within the limits of 12·6 and 15·2 D-glucose units (average 13·3) and the ECL(av.) values ranged from 7·9 to 9·3 units (average 8·7) (Table 2). A CL(av.) value of 15·2 D-glucose units for the glycogen from 7-week liver, although high, can be accounted for by the fact that there is an intrinsic error of ±1 D-glucose unit in the method employed.

Kitamura (1942) suggested that glycogen deposition first occurs in human foetal liver at 17 weeks and that the amount begins to increase sharply

Table 1. *Structures of human foetal-liver glycogens*

Foetal age (weeks)	Fresh wt. of liver sample (mg.)	Purity of glycogen (%)	Wt. of glycogen (mg.)	Glycogen recovered from liver (%)	CL(av.) by α-amylolysis	ECL(av.) by β-amylolysis	ICL(av.)
13½	420	51·0	1·65	0·39	14·1	9·2	3·9
16	846	45·2	1·95	0·23	13·0	10·2	1·8
17	929	58·0	2·26	0·24	13·5	8·7	3·8
18½	554	58·0	2·55	0·46	12·5	7·7	3·8
20	870	22·0	2·57	0·30	14·5	—	—
21½	465	27·2	2·23	0·48	13·7	9·1	3·6
21½	1567	68·0	3·13	0·20	13·6	8·0	4·6
23½	1944	60·0	4·68	0·24	14·1	9·0	4·1
25	246	83·5	1·75	0·71	14·0	8·7	4·3
26	180	66·3	3·05	1·70	13·5	8·4	4·1
26	28·33g.	86·6	320	1·13	13·9*	8·9	4·0

* CL(av.) by periodate oxidation = 13·4 D-glucose units.

Table 2. *Structures of liver glycogens obtained from children*

Age of patient	Fresh wt. of liver sample (mg.)	Purity of glycogen (%)	Wt. of glycogen (mg.)	Glycogen recovered from liver (%)	CL(av.) by α-amylolysis	ECL(av.) by β-amylolysis	ICL(av.)
6 weeks	50	45·0	1·4	2·80	—	*	—
7 weeks	566	62·5	4·5	0·80	15·2	9·2	5·0
8 weeks	44	57·3	2·0	4·55	12·6	8·9	2·7
12 weeks	1090	85·3	19·7	1·81	13·4	7·9	4·5
12 weeks	1298	69·7	15·9	1·23	12·9	8·3	3·6
14 weeks	359	67·6	4·3	1·20	13·9	9·3	3·6
20 months	189	45·0	1·4	0·74	13·1	9·2	2·9
28 months	235	98·6	13·2	5·62	12·7	8·6	3·1
11 years	502	76·6	8·4	1·67	12·9	8·2	3·7

* This sample gave 45% conversion into maltose (the normal range is 40–50% conversion).

Table 3. *Wavelengths of absorption maxima of glycogen–iodine complexes*

Source of liver glycogen	CL(av.) by periodate oxidation	CL(av.) by α-amylolysis	ECL(av.) by β-amylolysis	ICL(av.)	$\lambda_{max.}$ (mμ)
26-week human foetus	13·4	13·9	8·9	4·0	470
Baboon	13·3	13·3	8·3	4·0	435
Mouse	13·1	13·8	8·6	3·9	455
Rabbit	13·9	12·6	8·1	4·2	470

at 26 weeks. Claims have been made that glycogen is present in the liver as early as 6 weeks (Shapovalov, 1961, 1962), but both conclusions were based on histochemical studies. Mackay, Adams, Hertig & Danziger (1956) have, however, suggested that a 4-week foetal liver is almost ready to assume the function of glycogen storage. At this stage the yolk sac still fulfils many of the liver's functions, such as glycogen storage, but has begun to transfer its enzyme systems to the liver. Thus 5-nucleotidase and non-specific esterase begin to appear in the liver and decrease in activity in the yolk sac, and Mackay *et al.* (1956) suggest on this basis that the function of glycogen storage will soon be taken over by the liver. In contrast, Lelong, Rossier & Laumonier (1951) could not detect glycogen in human foetal liver, by histochemical techniques, until a few weeks before birth.

The methods employed in the present study are, of course, too insensitive to reveal very small amounts of glycogen, and we believe that the polysaccharide, and hence the enzyme system necessary for its synthesis, is present in foetal livers younger than 13½ weeks.

The stage at which glycogen storage begins to increase rapidly appears to bear some relation to the class of mammal. In rats, guinea pigs and rabbits (rodents) it occurs very late in gestation, i.e. after 90% (Jacquot, 1959), 80% (Ducommun-Lehmann, 1951) and 75% (Jost & Jacquot, 1955) respectively of the gestation period has elapsed. In primates it occurs at a much earlier stage, for example in man after 66% of gestation has elapsed, and in Rhesus monkeys (Shelley, 1960) glycogen deposition is already high at 113 days (67% of gestation). Ungulates vary widely, the rapid increase occurring after 88% of gestation in pigs (Mendel & Leavenworth, 1907; Aron, 1922) and 61% in sheep (Shelley, 1960). Since the pituitary and adrenal hormones and insulin stimulate glycogen synthesis, it appears that the hormonal systems of primates and probably ungulates mature earlier than those of the rodents. Newborn and full-term infants respond, by pituitary discharge of adrenocorticotrophic hormone, to adrenaline (Jailer, Wong & Engle, 1951), whereas rats and mice do not respond until the eighth and eleventh days of life respectively (Jailer, 1950; Thompson & Blount, 1954), but direct comparative evidence of hormonal activities during the foetal stage is lacking.

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The Effect of 4-Dimethylamino-3′-methylazobenzene on ^{14}C-Labelled Amino Acid Incorporation by Rat-Liver Polysome Preparations

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1. The incorporation of ^{14}C-labelled amino acids into polysomal protein was studied in a system consisting of polysomes and pH 5 enzyme obtained 4 and 40hr. after a single intraperitoneal injection of 4-dimethylamino-3′-methylazobenzene. Labelling of the polysome fraction of preparations of both the 4hr.-treated and 40hr.-treated rats was considerably higher than in the normal control. 2. In further experiments on protein synthesis by polysomes from azo-dye-treated rats, the effect of replacing pH 5 enzyme with cell sap was studied. Incorporation of ^{14}C-labelled amino acids into polysomal protein was similar to that of the control. 3. Aggregate size of polysomes obtained from rats injected previously with 4-dimethylamino-3′-methylazobenzene was studied by sucrose-gradient centrifugation. Polysomes prepared at 4hr. after azo-dye administration contained a considerable amount of large aggregates (approx. 700s), whereas at 40hr. after administration of the azo-dye the amount of large aggregates was less than in the control. 4. Determination of the ultraviolet spectra of polysome preparations from both normal and azo-dye-treated rats revealed no difference between the preparations. On the other hand, the ultraviolet spectra of cell-sap fractions from the different preparations showed that there is a definite shift in the absorption maximum from 272mμ (normal) to 267mμ, 40hr. after treatment, with an intermediate value of 270mμ for the preparation from 4hr.-treated rats. The absorption minimum changes from 250mμ (normal) to 245mμ for the preparation from 40hr.-treated rats.

In previous reports on the effect of 4-dimethylamino-3′-methylazobenzene on [^{14}C]leucine incorporation into protein by a system consisting of washed microsomes and pH 5 enzyme, it was shown that incorporation was stimulated at 40hr. after administration of the azo-dye (Hawtrey & Schirren, 1962*a*; Hawtrey, Schirren & Dijkstra, 1963). These findings have been confirmed independently by Daudel, Jacquier & Touplain (1965) with the carcinogenic azo-dyes 4-dimethylamino-3′-methylazobenzene and 4-dimethylaminoazobenzene. Of further interest was their observation that the non-carcinogenic azo-dye *p*-aminoazobenzene was without effect on protein synthesis.

In the experiments *in vitro* (Hawtrey *et al.* 1963), stimulation of [^{14}C]leucine incorporation in the treated system was dependent on both microsomes and pH 5 enzyme. When pH 5 enzyme was replaced by cell sap (supernatant remaining after removal of mitochondria), however, very little stimulation of incorporation was observed at 40hr. after dye administration, whereas at 4hr. after administration incorporation was inhibited by approx. 50%. In this connexion, a diffusible inhibitor of [^{14}C]-leucine incorporation was found in supernatant fractions from both normal and treated rats after removal of pH 5 enzyme.

Through the studies of Warner, Knopf & Rich (1963), Goodman & Rich (1963), Wettstein, Staehelin & Noll (1963), Noll, Staehelin & Wettstein (1963) and others, it is now established that protein synthesis is carried out by clusters or aggregates of ribosomes known as polysomes. In the rat-liver cell, polysomes are attached to lipoprotein membranes of the endoplasmic reticulum. This material, which is not homogeneous in that it contains a number of different elements, is obtained in the fraction sedimented at high speed (105000*g*) by differential centrifugation and is usually referred to as the microsomal fraction. It was considered of importance therefore to carry out experiments similar to those described by Hawtrey *et al.* (1963), in which, however, polysomes replaced the microsomal fraction. The results obtained would enable us to establish whether the previously observed effect of the azo-dye on microsomal protein syn-

thesis is located in the polysomes (ribosomes) or lipoprotein membrane of the microsome fraction.

In this paper we present results which appear to indicate that 4-dimethylamino-3′-methylazobenzene exerts some of its effects at the polysome level.

MATERIALS AND METHODS

Chemicals and enzymes. Both the source and the details of the use of the following substances were as described by Hawtrey *et al.* (1963): ATP (disodium salt), GSH, ribonuclease, GTP (disodium salt), 4-dimethylamino-3′-methylazobenzene (m.p. 120·4–121·2°), formic acid (98%), tris and sucrose. Phosphoenolpyruvate (tricyclohexylammonium salt) and pyruvate kinase were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Phosphoenolpyruvate was prepared immediately before use from the tricyclohexylammonium salt by dissolving in water without neutralization (pH 7·0). [We studied the effect of using the tricyclohexylammonium salt of phosphoenolpyruvate as compared with the potassium salt (obtained from the barium–silver salt) on protein synthesis, and found that it is inhibitory to a certain extent. However, no difference in inhibition was observed between incorporating systems from normal and azo-dye-treated rats.] The ^{14}C-labelled amino acids were used in the form of a yeast hydrolysate (850 μc/mg.) obtained from Schwarz Bio-Research Inc., Orangeburg, N.Y., U.S.A. It was dissolved in water, neutralized to pH 7·6, and stored at −15°. Sodium deoxycholate was supplied by General Biochemicals Inc., Chagrin Falls, Ohio, U.S.A. Bovine albumin (crystalline) and yeast RNA (sodium salt) were obtained from the Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.

Preparation of cell particles and enzyme fractions. Female rats (100–120g.) were injected intraperitoneally with 4-dimethylamino-3′-methylazobenzene (50mg./250g. body wt.) dissolved in 2·0ml. of olive oil and killed at 4 and 40hr. thereafter. Animals killed 40hr. later were deprived of food 24hr. before death. This starvation period was also applied to the normal control animals, but the rats injected 4hr. before death were starved for 20hr. before injection.

Polysomes. Rat-liver homogenates were prepared in medium A [containing (final concentrations): sucrose (0·25M), $MgCl_2$ (5mM), KCl (25mM) and tris–HCl buffer, pH 7·6 (50mM)], as described by Hawtrey *et al.* (1963). Nuclei and cell debris were removed by centrifuging at 1400*g* for 10min. (all *g* values are given for the bottom of the centrifuge tube), and the resulting supernatant was centrifuged at 14370*g* in the Spinco model L centrifuge (no. 30 rotor) for 20min. to remove mitochondria. The supernatant after sedimenting mitochondria was used for the preparation of polysomes, which was carried out essentially by the method of Wettstein *et al.* (1963). In applying this method to centrifuge tubes of the no. 30 rotor, the following procedure was adopted: Supernatant after sedimenting mitochondria (13ml.) was mixed with 1·5ml. of 13% (w/v) sodium deoxycholate (freshly prepared in water), and allowed to stand in ice for 5–7min. before carefully layering on to a preformed double layer consisting of 9ml. of 2M-sucrose (bottom) and 12ml. of 0·5M-sucrose (upper). Both layers contained tris-HCl buffer, pH 7·6 (50mM), KCl (25mM) and $MgCl_2$ (5mM). Centrifugation was carried out at 99000*g* (no. 30 rotor) for 240min. After centrifugation, the solution above the polysomes was carefully removed by means of a large Pasteur pipette, and the sides of the tube were carefully wiped with filter paper. The pellet of polysomes was resuspended in 1·0ml. of medium A by means of a glass rod with gentle stirring, and the resulting suspension allowed to stand at 0° for 1hr. with occasional stirring. It was then centrifuged at 2000rev./min. (1400*g*) for 10min. to remove aggregated and denatured material. The final clear suspension of polysomes was kept at 0°.

Cell sap. This was prepared from a portion of the mitochondrial supernatant by centrifuging at 105000*g* for 60min.

pH 5 fraction. This was prepared from a sample of the cell sap as described by Hawtrey *et al.* (1963), except that the final suspension of washed pH 5 enzymes in medium A was centrifuged at 20000*g* for 10min. (no. 40 rotor) to remove denatured material.

Preparation of sucrose gradients. Linear sucrose gradients (15–30%, w/w) were prepared in no. 25 rotor tubes by using a modified mixing apparatus of the type described by Britten & Roberts (1960). Sucrose-gradient centrifugation was carried out in the no. SW 25.1 swinging-bucket rotor at 25000 rev./min. (90000*g*) for the times given in the text. All gradients were run at a 20°F temperature setting.

System for incorporation of ^{14}C-labelled amino acids into polysomes. The reaction medium used for incorporation studies contained: tris–HCl buffer, pH 7·6 (final concn. 50mM); KCl (final conc. 25mM); $MgCl_2$ (final concn. 8·5mM); sucrose (final concn. 0·25M); ATP (potassium salt), pH 7·6 (1·25 μmoles); GTP (potassium salt), pH 7·6 (0·5 μmole); phosphoenolpyruvate (tricyclohexylammonium salt) (10 μmoles); phosphoenolpyruvate kinase (60 μg.); ^{14}C-labelled yeast hydrolysate of specific activity 850 μc/mg. (1·4 μc); polysomes (0·8–2·0mg. of RNA); cell sap (4–6mg. of protein) or pH 5 fraction (2·5–5·0mg. of protein). The final reaction volume was 1·3ml. Incubations were carried out in test tubes at 37° for various times. In agreement with Sargent & Campbell (1965), the tricyclohexylammonium salt of phosphoenolpyruvate partly inhibited protein synthesis, but the use of this salt in no way invalidated the present results (see above). In experiments where the reaction mixture was to be analysed by sucrose-gradient centrifugation, it was first cooled in ice for 15min., then carefully layered on to a precooled sucrose gradient. Centrifugation was then carried out as described above. Tubes were punctured with a no. 21 syringe needle and 13-drop fractions collected (approx. 1ml.). Each fraction was then diluted by the addition of 1ml. of water and its extinction at 260mμ measured. The same fraction was later analysed for the incorporation of ^{14}C-labelled amino acids into protein as described below.

Isolation of ^{14}C-labelled proteins. To each fraction (2ml.) from a sucrose gradient was added 0·1ml. of a solution containing 0·25mg. of yeast RNA and 0·5mg. of albumin (carrier), followed by 0·2ml. of 60% (w/v) trichloroacetic acid. After mixing, the tubes were allowed to stand for 24–36hr. at 4°. This procedure allows hydrolysis of the transfer RNA with 90% release of labile counts (^{14}C-labelled amino acids attached to transfer RNA). The transfer RNA from both normal and azo-dye-treated rats was broken down by identical amounts. The resulting precipitates were washed successively three times with 5% (w/v) trichloroacetic acid (5ml./tube). During the first

wash, 0·1 ml. of a complete unlabelled amino acid mixture was added to each tube. After washing, the precipitates were dissolved in 0·5 ml. of 2·5% (w/v) NH_3 and plated. The planchets were dried under infrared lamps. Samples were counted with a Philips electronic counter (tube PW 41491) and appropriate corrections for thickness and background made.

Protein and RNA. These were determined as described by Hawtrey *et al.* (1963).

RESULTS

Incorporation of ^{14}C-labelled amino acids into polysomes at 40 hr. after the administration of 4-dimethylamino-3′-methylazobenzene. In the system consisting of polysomes and cell sap from rats injected with azo-dye 40 hr. previously, polysomes are labelled after a 10 min. incubation to an extent similar to that of the normal control (Fig. 1). In this and all subsequent experiments labelling of the polysomes is reported as total counts and as such these are not specific activities. In experiments with incubation for 20 min. (Fig. 2), the treated and normal polysomes are again labelled to similar extents.

Further experimental work was carried out with a system consisting of polysomes but with pH 5 enzyme instead of cell sap. Results of experiments at 40 hr. after azo-dye administration are shown in Fig. 3. Incorporation of ^{14}C-labelled amino acids into both polysomes and supernatant protein occurs to a greater extent in the system with fractions from treated rats than in the system with fractions from normal rats: total counts of polysomes from treated rats, 1886 counts/min.; supernatant protein from treated rats, 2730 counts/min.; polysomes from normal rats, 1208 counts/min.; supernatant protein from normal rats, 1945 counts/min. Equal amounts of both polysomes and cell sap or pH 5

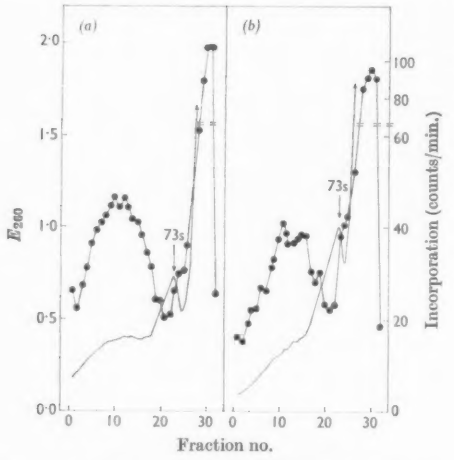

Fig. 1. Sucrose-gradient centrifugation of polysomes labelled with ^{14}C-labelled amino acids in the presence of cell sap. Polysomes were prepared from livers of both normal and azo-dye-treated rats. 4-Dimethylamino-3′-methylazobenzene (50 mg./250 g. body wt.) was administered by intraperitoneal injection 40 hr. previously. Labelling of the polysomes was carried out in the following reaction medium: tris–HCl buffer, pH 7·6 (final concn. 50 mM); KCl (final concn. 25 mM); $MgCl_2$ (final concn. 8·5 mM); sucrose (final concn. 0·25 M); ATP (1·25 μmoles); GTP (0·5 μmole); phosphoenolpyruvate (tricyclohexylammonium salt) (10 μmoles); phosphoenolpyruvate kinase (60 μg.); ^{14}C-labelled yeast hydrolysate (1·4 μc); polysomes (1·2 mg. of RNA) from normal or treated rats; cell sap (4·9 mg. of protein) from normal rats or cell sap (5·0 mg. of protein) from treated rats. The final reaction volume was 1·3 ml. Reactions were run at 37° for 10 min. Centrifugation was carried out at 25000 rev./min. (no. 25.1 rotor) for 150 min. [gradients were 15–30% (w/w) sucrose in the medium A buffer system]. (*a*) Polysomes from normal rats plus cell sap from normal rats; (*b*) polysomes from treated rats plus cell sap from treated rats. —, E_{260}; ●, incorporation (stable counts).

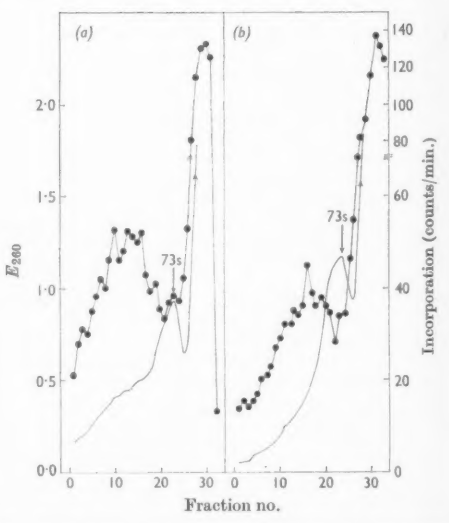

Fig. 2. Sucrose-gradient centrifugation of polysomes labelled with ^{14}C-labelled amino acids in the presence of cell sap for 20 min. at 37°. Incubation conditions were as described for Fig. 1. Centrifugation was carried out for 150 min. at 25000 rev./min. (*a*) Polysomes from normal rats plus cell sap from normal rats; (*b*) polysomes from treated rats plus cell sap from treated rats. —, E_{260}; ●, incorporation (stable counts). The azo-dye was administered 40 hr. previously as described for Fig. 1.

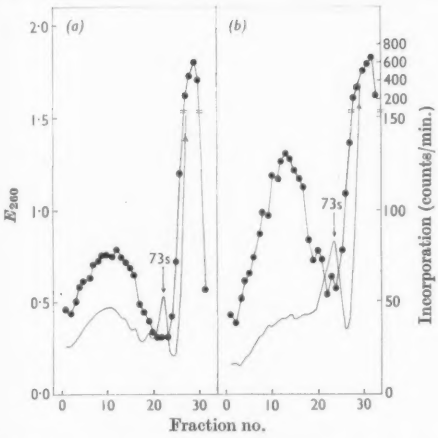

Fig. 3. Analysis of polysomes (prelabelled in the presence of pH 5 enzyme) by sucrose-gradient centrifugation. The reaction medium and conditions of labelling and centrifuging were as described in Fig. 1. Azo-dye was administered to rats 40 hr. previously. Polysomes (1·2 mg. of RNA) from normal or treated rats were incubated with pH 5 enzyme (2·7 mg. of protein) from normal or treated rats for 10 min. at 37°. Centrifugation was at 25 000 rev./min. for 150 min. (*a*) Polysomes from normal rats plus pH 5 enzyme from normal rats; (*b*) polysomes from treated rats plus pH 5 enzyme from treated rats. —, E_{260}; ●, incorporation (stable counts).

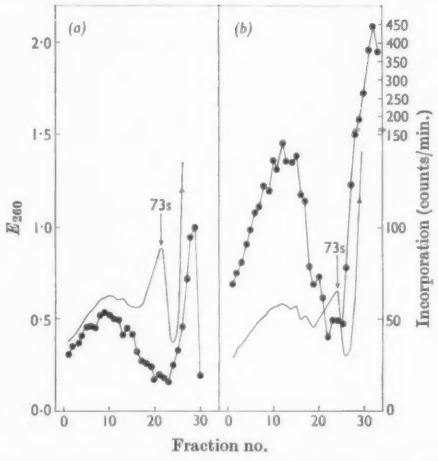

Fig. 4. Sucrose-gradient centrifugation of polysomes prelabelled either in the presence of cell sap of pH 5 enzyme. 4-Dimethylamino-3′-methylazobenzene was administered by intraperitoneal injection 4 hr. previously. Conditions for the experiment were as described in Fig. 1. Polysomes (1·4 mg. of RNA) from treated rats were incubated with cell sap (5·2 mg. of protein) from treated rats or pH 5 enzyme (2·8 mg. of protein) from treated rats for 10 min. at 37°. (*a*) Polysomes from treated rats plus cell sap from treated rats; (*b*) polysomes from treated rats plus pH 5 enzyme from treated rats. —, E_{260}; ●, incorporation (stable counts).

enzyme were used whenever a comparison was made between systems of fractions from normal and treated rats.

Incorporation of ^{14}C*-labelled amino acids into polysomes at 4 hr. after the administration of 4-dimethylamino-3′-methylazobenzene.* Results presented in Fig. 4 show the incorporation of ^{14}C-labelled acids into polysomes and supernatant protein in systems consisting of polysomes from treated rats plus cell sap from treated rats (Fig. 4*a*), and polysomes from treated rats plus pH 5 enzyme from treated rats (Fig. 4*b*) after 10 min. of incubation at 37°. The labelling of polysomes and supernatant protein of the system containing cell sap as a source of activating and transfer enzymes is low when compared with the system in which pH 5 enzyme replaces cell sap. Total counts for different polysome and supernatant proteins are as follows: in Fig. 4(*a*), polysomes, 799 counts/min.; supernatant protein, 365 counts/min.; in Fig. 4(*b*), polysomes, 2044 counts/min.; supernatant protein, 1652 counts/min.

The incorporation of the polysomes from rats treated 4 hr. previously supplemented with cell sap is similar to that found with the polysomes from 40 hr.-treated and normal rats, the results of which are shown in Fig. 1. There was high labelling of polysomes in the system consisting of polysomes and pH 5 enzyme (Fig. 4*b*), which is similar to that of the system of fractions from 40 hr.-treated rats, but greater than that of the system of fractions from normal rats (Fig. 3). With regard to the polysome–pH 5 enzyme system, with both fractions from 4 hr.-treated rats (Fig. 4*b*), breakdown of polysomes is not extensive, yet the labelling of both the polysomes and supernatant protein is high. On the other hand, there appears to be a more extensive breakdown of polysomes in the system where pH 5 enzyme is replaced by cell sap (Fig. 4*a*), as shown by the height of the 73 s monomer peak, and labelling is relatively small.

Size and stability of polysomes obtained from rats injected 4 and 40 hr. previously with 4-dimethylamino-3′-methylazobenzene. In view of the results obtained above on the stimulation of incorporation of ^{14}C-labelled amino acids into polysomal and supernatant protein of the systems of fractions from azo-dye-treated rats, it was considered important to study the size and stability of the different polysome preparations.

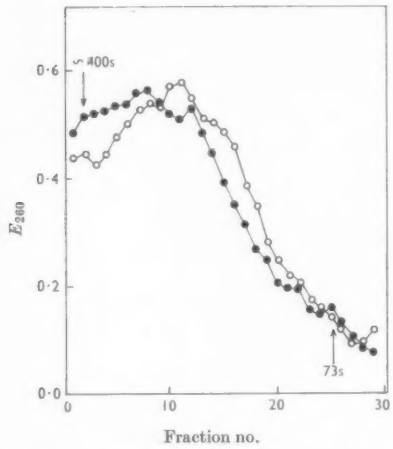

Fig. 5. Extinction profiles of polysome preparations after sucrose-gradient centrifugation at 25000 rev./min. (no. 25.1 rotor) for 105 min. ●, Polysomes from normal rats; ○, polysomes from 40 hr.-treated rats.

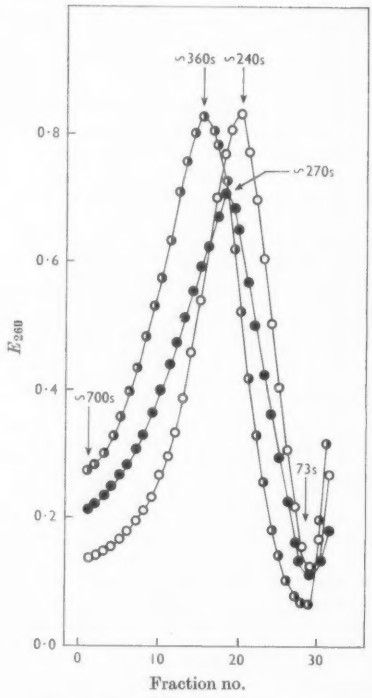

Fig. 6. Sucrose-gradient centrifugation of different polysome preparations. Centrifugation was carried out at 25000 rev./min. for 50 min. ◑, Polysomes from 4 hr.-treated rats; ●, polysomes from normal rats; ○, polysomes from 40 hr.-treated rats. Sucrose gradients were 15–30% (w/w) in the buffer system of medium A.

Results in Fig. 5 show the extinction profiles of polysome preparations after sucrose-gradient centrifugation at 25000 rev./min. for 105 min. The polysome preparation from 40 hr.-treated rats has a peak to the right of the normal polysome peak, suggesting that it has a higher proportion of smaller aggregates than the normal.

To obtain a clearer picture of the polysome distribution in the different preparations, centrifugation was carried out for the shorter time of 50 min. (Fig. 6). The polysomes obtained at 4 hr. after azo-dye treatment contain a higher percentage of large aggregates (approx. 700 s) than the preparations from either the normal or 40 hr.-treated rats.

Stability of the polysome preparations from normal and treated rats was studied by allowing the polysomes (in medium A) to age for 1 week at 0°. Thereafter, they were examined as to their aggregate size by sucrose-gradient centrifugation. The polysomes from 4 hr.-treated rats were broken down to smaller aggregates to a greater extent than the preparation made 40 hr. after azo-dye administration. Results intermediate between those obtained with the preparations from treated rats were observed with the polysomes from normal rats.

Ultraviolet spectra of enzyme and polysome fractions. During the present work, ultraviolet measurements were always carried out on both the enzymes and different polysome preparations. It was noticed that the E_{260}/E_{280} ratio varied between different samples of cell sap taken at different times after administration of the azo-dye. Hence complete ultraviolet spectra of the different cell-sap fractions were determined (Fig. 7). The results show clearly a progressive shift of the absorption maximum from 272 mμ (normal) through 270 mμ (4 hr.-treated) to 267 mμ (40 hr.-treated). There is also a simultaneous shift in the absorption minimum (Fig. 7).

Determination of the ultraviolet spectra of the polysome preparations from normal and azo-dye treated rats, on the other hand, revealed little difference between their spectral curves (Fig. 8).

DISCUSSION

Polysomes prepared at 4 and 40 hr. after a single intraperitoneal injection of 4-dimethylamino-3′-methylazobenzene exhibited a higher degree of

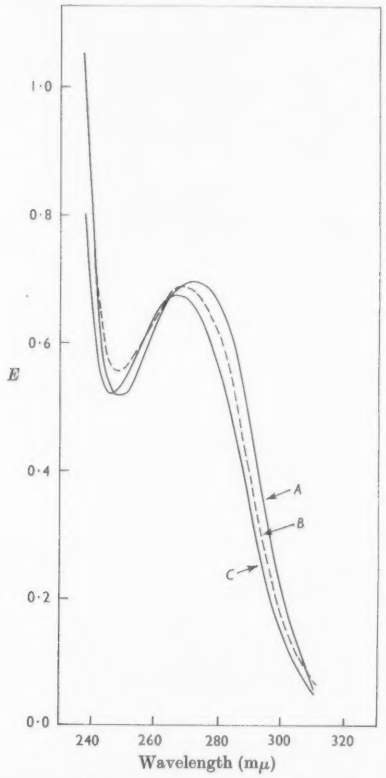

Fig. 7. Ultraviolet spectra of different cell-sap fractions. All spectra were determined in medium A. Each cuvette contained 1·6mg. of protein. *A*, Cell sap from normal rats; *B*, cell sap from 4hr.-treated rats; *C*, cell sap from 40hr.-treated rats.

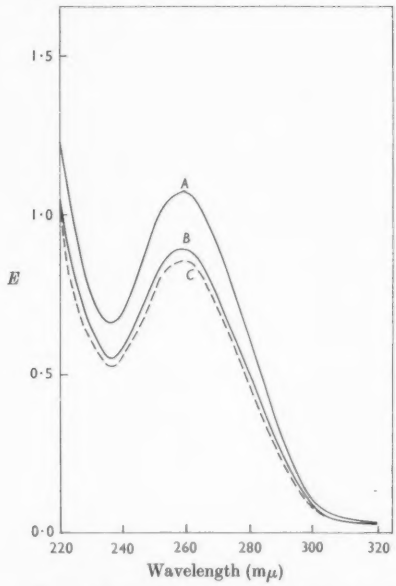

Fig. 8. Ultraviolet spectra of different polysome preparations. All spectra were determined in medium A. *A*, Polysomes (0·25mg. of ribonucleoprotein) from normal rats; *B*, polysomes (0·20mg. of ribonucleoprotein) from 40hr.-treated rats; *C*, polysomes (0·19mg. of ribonucleoprotein) from 4hr.-treated rats.

labelling in comparison with polysomes from normal rats, when each incorporating system was supplemented with its own pH5 enzyme fraction (Figs. 3 and 4). In particular, polysomes prepared at 4hr. after injection were labelled to the greatest extent. This latter finding is in contrast with previous results (Hawtrey, *et al.* 1963) on the microsome–pH5 enzyme system obtained at 4hr. after azo-dye administration, where incorporation was little different from that observed for the control. The present results, and those obtained previously, suggest therefore that the lipoprotein membrane of the microsome complex may have some controlling influence on protein synthesis. Further, the results appear to indicate that, at 4hr. after administration, 4-dimethylamino-3′-methylazobenzene interferes with certain functions in the lipoprotein membrane which are associated with protein synthesis. In this connexion, Hoagland, Scornik & Pfefferkorn (1964) have demonstrated the presence in rat-liver microsomes of a heat-labile inhibitory factor of protein synthesis. It is tempting to suggest that this factor is present in the membrane of the microsome complex. Hawtrey & Schirren (1962*b*) have reported on the inhibition of [^{14}C]leucine incorporation into rat-liver ribosomes (nucleoprotein particles) by a lipoprotein fraction (membrane) obtained from microsomes by iso-octane extraction. Further detailed study on the membrane fraction of rat-liver microsomes is necessary to substantiate these ideas.

Little difference was observed in the labelling patterns of polysome preparations from normal and treated rats when supplemented with cell sap as a source of enzymes (Figs. 1 and 2). In particular, polysomes prepared 4hr. after treatment were labelled to an extent similar to that of the normal control preparation. This result is also in contrast with previous work (Hawtrey *et al.* 1963) where considerable inhibition of incorporation was

Table 1. *Summary of the effects of 4-dimethylamino-3′-methylazobenzene on protein synthesis by rat-liver microsomes and polysomes*

Results on the polysomes are taken from the present work, and those on the microsomes are from a previous investigation (Hawtrey, *et al.* 1963).

	Effect of 4-dimethylamino-3′-methylazobenzene on protein synthesis	
Time after azo-dye treatment.....	4 hr.	40 hr.
System		
Microsomes plus pH 5 enzyme	Normal	Stimulation
Microsomes plus cell sap	Inhibition	Normal
Polysomes plus pH 5 enzyme	Stimulation	Stimulation
Polysomes plus cell sap	Normal	Normal

observed in a system consisting of microsomes and cell sap (supernatant remaining after removal of mitochondria) obtained at 4 hr. after azo-dye administration. At present, it is difficult to interpret exactly how the azo-dye is affecting protein synthesis, but in terms of the experimental findings of this and a previous paper (Hawtrey *et al.* 1963) we can state that at the early time of 4 hr. after azo-dye administration the rate and amount of protein synthesis is somehow affected by the lipoprotein membrane of the microsomal fraction, and that the effect is influenced by the presence of cell sap or pH 5 enzyme. To describe the effects of the azo-dye on protein synthesis in the various systems studied, we have very briefly summarized the results of the present and previous work (Hawtrey *et al.* 1963) in Table 1. At 40 hr. after azo-dye administration it is clear that stimulation of incorporation into either microsomes or polysomes is brought about through the use of pH 5 enzyme.

The change in the ultraviolet-absorption maximum of the cell sap after 4-dimethylamino-3′-methylazobenzene administration is noteworthy. A progressive shift from 272 mμ (normal) to 267 mμ 40 hr. after treatment was observed (Fig. 7), and is probably related to some change in the RNA/protein ratio of the cell sap or increase in nucleotide material.

The present work shows that 4-dimethylamino-3′-methylazobenzene can exert effects on protein synthesis in rat liver at the level of both the polysome and lipoprotein membrane of the microsome fraction.

The authors are indebted to Mr Philip Nourse for his excellent assistance with certain phases of the work, and to Dr H. M. Schwartz for her interest.

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Toxic Fluorine Compounds

THE USE OF THE ω-FLUORINE ATOM IN THE STUDY OF THE METABOLISM OF FATTY ACIDS CONTAINING ETHYL, *n*-PROPYL AND ISOPROPYL BRANCHES

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1. Some ω-fluoroalkanoic acids containing a branched ethyl, *n*-propyl or isopropyl group in the α- or β-position have been prepared. 2. A study of the toxicity of these acids revealed that the ethyl group partially inhibited, and the *n*-propyl and isopropyl groups completely inhibited, the normal processes of oxidative degradation.

Pattison & Buchanan (1964) described the value of the ω-fluorine atom in elucidating the catabolism of fatty acids containing branched methyl groups; the rationale resided in the characteristic toxicological pattern of the homologous series of ω-fluoroalkanoic acids, $F\cdot[CH_2]_n\cdot CO_2H$. The method appears to be both general and simple, merely involving the preparation of a few compounds and the determination of their toxicity.

The ω-fluorine atom, in its role as a 'tag' (Pattison, 1959), offers several advantages over the more usual approach of isolating breakdown products after the administration of appropriate prototypes to animals. First, the determination of toxicity is simpler and less time-consuming than the isolation and estimation of individual catabolites. Secondly, the fluorine atom, being situated in the ω-position, eliminates the competitive biochemical route of ω-oxidation, allowing full attention to be directed to the structural features under study. Thirdly, the atomic dimensions of the fluorine atom, approximating those of a hydrogen atom, result in the ω-fluoro derivatives being structurally very similar to the parent compounds. The ω-fluorine atom is thus sterically less grotesque than the larger groups, e.g. phenyl, that were used in the early studies of β-oxidation (Knoop, 1904). Fourthly, small quantities of material may be administered, thus avoiding massive doses of prototypes, which, by flooding the system, can readily produce atypical results and from which individual catabolites are frequently isolated in such low percentage yield as to represent an insignificant fraction of the total products of metabolism.

Since previous results on methyl branching showed that *gem*-dimethyl groups in any position inhibited β-oxidation, and it has been well established that β-oxidation occurs up to the vicinity of the branch, no matter where the original branch may be located (Weitzel, 1951; Pattison & Woolford, 1957), the problem reduces to the metabolism of the α- and β-monoalkyl derivatives.

The ω-fluoro acids were prepared by the general procedures previously used for some of the ω-fluoro branched-methyl derivatives (Pattison & Woolford, 1957; Pattison & Buchanan, 1964).

α-Alkyl-ω-fluoroalkanoic acids were obtained by malonate condensations:

$$F\cdot[CH_2]_n\cdot X + NaCR(CO_2Et)_2 \rightarrow F\cdot[CH_2]_n\cdot CR(CO_2Et)_2 \xrightarrow[-CO_2]{\text{Hydrolysis}} F\cdot[CH_2]_n\cdot CHR\cdot CO_2H$$

($n = 5, 6, 7$; R = Et, Pr^n, Pr^i)

β-Alkyl-ω-fluoroalkanoic acids were obtained from the corresponding α-alkyl-ω-fluoroalkanoic acids by Arndt–Eistert homologation:

$$F\cdot[CH_2]_n\cdot CHR\cdot CO_2H \xrightarrow{SOCl_2} F\cdot[CH_2]_n\cdot CHR\cdot COCl \xrightarrow{CH_2N_2} F\cdot[CH_2]_n\cdot CHR\cdot CO\cdot CHN_2 \xrightarrow[Ag_2O]{H_2O} F\cdot[CH_2]_n\cdot CHR\cdot CH_2\cdot CO_2H$$

($n = 5, 6$; R = Et, Pr^n, Pr^i)

RESULTS AND DISCUSSION

The toxicity determinations were carried out on male mice weighing 28–32g. from a Wistar strain colony maintained at Suffield Experimental Station, Ralston, Alberta, Canada, since 1946. The compounds were administered by intraperitoneal injection in propylene glycol; the total volume never exceeded 0·1ml. The LD_{50} values and 95% fiducial limits were calculated by the method of

Table 1. *Toxicities of branched ω-fluoroalkanoic acids*

The 95% confidence limits are given in parentheses.

Compounds	LD_{50} found (mg./kg.) R=H*	R=Me†	R=Et	R=Pr^n	R=Pr^i
8-Fluoro-octanoic acids					
$F\cdot[CH_2]_6\cdot CHR\cdot CO_2H$ (α-R)	0·64	1·1‡	5·5 (3·9–7·8)	> 75§	> 100
$F\cdot[CH_2]_5\cdot CHR\cdot CH_2\cdot CO_2H$ (β-R)	0·64	> 100	> 100	> 100	> 100
9-Fluorononanoic acids					
$F\cdot[CH_2]_7\cdot CHR\cdot CO_2H$ (α-R)	> 100	> 100	> 100	> 100	> 100
$F\cdot[CH_2]_6\cdot CHR\cdot CH_2\cdot CO_2H$ (β-R)	> 100	4·5 (1·80–11·2)	25·5 (17·0–38·3)	> 100	> 100

* Pattison, Hunt & Stothers (1956).
† Pattison & Buchanan (1964).
‡ Estimated value.
§ Mortality figures: at 100 mg./kg., 3/5; at 75 mg./kg., 0/5.

Litchfield & Wilcoxon (1949). The results are shown in Table 1.

Some α-branched ω-fluoroheptanoic acids ($F\cdot[CH_2]_5\cdot CHR\cdot CO_2H$; R = Et, Pr^n and Pr^i) were prepared as intermediates in the synthesis of the β-alkyl-ω-fluoro-octanoic acids. They were non-toxic (LD_{50} > 100 mg./kg. in all cases); the unbranched 7-fluoroheptanoic acid was also non-toxic (LD_{50} > 80 mg./kg.).

The toxicity results shown in Table 1 include the previously reported values for the parent unbranched ω-fluoroalkanoic acids (Pattison, Hunt & Stothers, 1956) and the corresponding acids containing a branched methyl group (Pattison & Buchanan, 1964).

For the 8-fluoro-octanoic acids containing an α-alkyl branch, it is clear that the α-methyl group offers no hindrance to the normal β-oxidation process. When the branched group is increased in size from methyl to ethyl, the same result is apparent qualitatively, but quantitatively the acid is fivefold less active than the branched methyl analogue. This is probably a steric effect, whereby the larger groups increasingly inhibit the action of the enzyme.

The 7-fluoroheptanoic acids and 9-fluorononanoic acids containing an α-alkyl branch are all non-toxic for one of two reasons: first, the acids containing a methyl or an ethyl branch allow normal β-oxidation to occur but give rise to a non-toxic end product; secondly, β-oxidation of the acids containing a larger branch is inhibited on steric grounds.

A similar pattern can be seen with the 9-fluorononanoic acids containing a β-alkyl branch. The β-methyl group causes complete reversal of toxicity, as explained by Pattison & Buchanan (1964). The β-ethyl group mimics this to a 5·5-fold smaller extent. A steric effect again provides the most likely explanation. As before, a further increase in size of the branch to *n*-propyl and isopropyl results in the expected loss of toxic properties.

The 8-fluoro-octanoic acids containing a β-alkyl branch are all non-toxic, as expected, either because a non-toxic catabolite is formed (R = Me or Et) or because degradation is inhibited (R = Pr^n or Pr^i). The fact that 8-fluoro-2-*n*-propyloctanoic acid shows some slight toxicity suggests two possibilities: that some ω-oxidation of the *n*-propyl branch may be occurring, which by subsequent β-oxidation and decarboxylation might give rise to traces of the toxic 8-fluoro-octanoic acid; or that the *n*-propyl group may be a less effective steric inhibitor (relative to the isopropyl group) in the formation of the enzyme–substrate complex.

In summary, a branched ethyl group, being larger, gives results that are qualitatively similar to those obtained with the branched methyl group, whereas still larger groups, such as *n*-propyl and isopropyl, in any position cause complete inhibition of β-oxidation. It is perhaps significant that the 'inhibiting effect' of the ethyl group relative to the methyl group, in both the α- and β-positions, is approximately the same; this consistency provides additional support for the explanation of steric inhibition. Groups still larger than propyl may also inhibit β-oxidation unless some competitive reaction, such as ω-oxidation of the branch, can occur.

Williams (1959) has surveyed much of the earlier work relating to branched acids. In general, acids containing branches larger than methyl in the α- or β-position are metabolized only by ω-oxidation; in no instance has β-oxidation removed the branched group (Weitzel, 1951; Keil, 1947). Because ω-oxidation is ruled out in the present work by the presence of the ω-fluorine atom, it might be supposed that the tendency for β-oxidation to occur would be correspondingly enhanced. Hence the observed lack of toxicity of

Table 2. *Physical constants and analytical results of ω-fluoroalkanoic acids*

Compound	Boiling point (°C)	Boiling point (mm. Hg)	n_D^{25}	Analytical results: C (%) Calc.	C (%) Found	H (%) Calc.	H (%) Found
Alkyl-ω-fluoroalkylmalonic acid esters							
F·[CH$_2$]$_5$·CEt(CO$_2$Et)$_2$	106	0·25	1·4296	60·9	61·1	9·1	9·4
F·[CH$_2$]$_6$·CEt(CO$_2$Et)$_2$	135	2·00	1·4320	62·0	62·2	9·4	9·4
F·[CH$_2$]$_7$·CEt(CO$_2$Et)$_2$	119	0·35	1·4322	63·1	63·0	9·6	9·6
F·[CH$_2$]$_5$·CPr^n(CO$_2$Et)$_2$	107	0·80	1·4317	62·0	62·0	9·4	9·2
F·[CH$_2$]$_6$·CPr^n(CO$_2$Et)$_2$	106	0·20	1·4330	63·1	63·4	9·6	9·4
F·[CH$_2$]$_7$·CPr^n(CO$_2$Et)$_2$	126	0·30	1·4351	64·1	64·4	9·8	9·9
F·[CH$_2$]$_5$·CPr^i(CO$_2$Et)$_2$	100	0·20	1·4352	62·0	62·2	9·4	9·2
F·[CH$_2$]$_6$·CPr^i(CO$_2$Et)$_2$	108	0·25	1·4366	63·1	63·4	9·6	9·8
F·[CH$_2$]$_7$·CPr^i(CO$_2$Me)$_2$	122	0·55	1·4405	62·0	62·2	9·4	9·5
α-Alkyl-ω-fluoroalkanoic acids							
F·[CH$_2$]$_5$·CHEt·CO$_2$H	120	1·50	1·4271	61·3	61·5	9·7	9·6
F·[CH$_2$]$_6$·CHEt·CO$_2$H	135	0·30	1·4297	63·1	63·0	10·1	9·9
F·[CH$_2$]$_7$·CHEt·CO$_2$H	126	0·20	1·4326	64·7	64·6	10·4	10·3
F·[CH$_2$]$_5$·CHPr^n·CO$_2$H	108	0·30	1·4301	63·1	63·3	10·1	10·1
F·[CH$_2$]$_6$·CHPr^n·CO$_2$H	126	0·55	1·4334	64·7	65·0	10·4	10·4
F·[CH$_2$]$_7$·CHPr^n·CO$_2$H	132	0·55	1·4353	66·0	66·2	10·6	10·7
F·[CH$_2$]$_5$·CHPr^i·CO$_2$H	107	0·30	1·4358	63·1	63·2	10·1	9·9
F·[CH$_2$]$_6$·CHPr^i·CO$_2$H	122	0·50	1·4353	64·7	65·0	10·4	10·5
F·[CH$_2$]$_7$·CHPr^i·CO$_2$H	121	0·40	1·4357	66·0	66·3	10·6	10·4
β-Alkyl-ω-fluoroalkanoic acids							
F·[CH$_2$]$_5$·CHEt·CH$_2$·CO$_2$H	118	0·80	1·4359	63·1	63·3	10·1	10·1
F·[CH$_2$]$_6$·CHEt·CH$_2$·CO$_2$H	125	0·50	1·4372	64·7	64·5	10·4	10·2
F·[CH$_2$]$_5$·CHPr^n·CH$_2$·CO$_2$H	108	0·20	1·4360	64·7	64·8	10·4	10·4
F·[CH$_2$]$_6$·CHPr^n·CH$_2$·CO$_2$H	127	0·33	1·4381	66·0	66·2	10·6	10·6
F·[CH$_2$]$_5$·CHPr^i·CH$_2$·CO$_2$H	118	0·30	1·4414	64·7	64·9	10·4	10·4
F·[CH$_2$]$_6$·CHPr^i·CH$_2$·CO$_2$H	120	0·30	1·4401	66·0	66·0	10·6	10·7

the *n*-propyl and isopropyl derivatives clearly shows that β-oxidation is effectively blocked by such branched groups.

EXPERIMENTAL

Refractive indices were measured on a thermostatically controlled Bausch and Lomb refractometer. Analyses were performed by the Schwarzkopf Microanalytical Laboratory, Woodside, 77, New York, N.Y., U.S.A. Fractional distillation was carried out on a 23-plate platinum-plated spinning-band column (18in.). Physical constants and analytical results are listed in Table 2.

α-Alkyl-ω-fluoroalkanoic acids

Alkyl-ω-fluoroalkylmalonic acid esters. Diethyl 5-fluoropentyl-n-propylmalonate was prepared as follows. Diethyl *n*-propylmalonate (41·7g., 0·207mole) was added over a period of 5min. to a stirred solution of sodium (7·2g., 0·313g.atom) in ethanol (120ml.). The solution was stirred and heated under reflux for a further 30min., and then 5-fluoropentyl bromide (Pattison & Howell, 1956) (35·0g., 0·207mole) was run in over 10min. The resultant mixture was refluxed in an oil bath for 4hr.; the reflux condenser was then set for distillation, and the bulk of the solvent was distilled off. Ice–water (250ml.) was added, and the ester layer was separated and added to two ether extracts of the aqueous layer. The ethereal solution was dried over $CaCl_2$ and distilled. The yield of product, after fractionation, was 41·6g. (77%).

The following were obtained in the same way: *diethyl ethyl-5-fluoropentylmalonate* (72%), *diethyl ethyl-6-fluorohexylmalonate* (66%), *diethyl ethyl-7-fluoroheptylmalonate* (56%), *diethyl 6-fluorohexyl-n-propylmalonate* (62%) and *diethyl 7-fluoroheptyl-n-propylmalonate* (59%).

Diethyl 5-fluoropentylisopropylmalonate was prepared as follows. A mixture of NaH (6·3g. of a 56·5% dispersion in mineral oil, 0·149mole) and dry dimethylformamide (110ml.) was placed in a 250ml. flask fitted with a stirrer, a reflux condenser protected by a drying tube, and a dropping funnel. Diethyl isopropylmalonate (30·0g., 0·149mole) was added over a period of 15min. and the solution was stirred until the evolution of hydrogen had ceased (about 1hr.). 5-Fluoropentyl bromide (Pattison & Howell, 1956) (25·1g., 0·149mole) was added slowly and the mixture was heated to 85° in an oil bath and kept at that temperature until the pH had become steady at approx. 6·5 (about 43hr.). The solution was poured into ice–water (400ml.). The resultant mixture was extracted with ether and the extracts were washed with water and dried over $CaSO_4$. Distillation and fractionation gave 35·8g. (83%) of the pure product.

The following were obtained in the same way: *diethyl 6-fluorohexylisopropylmalonate* (60%) and *dimethyl 7-fluoroheptylisopropylmalonate* (67%).

α-Alkyl-ω-fluoroalkanoic acids. *2-Ethyl-8-fluoro-octanoic acid* was prepared as follows. Diethyl ethyl-6-fluorohexylmalonate (31·8g., 0·11mole), ethanol (50ml.) and 10% (w/v) NaOH solution (250ml.) were gently heated under reflux until a homogeneous solution was obtained (3–4hr.). The ethanol was removed by distillation and the residue was cooled to room temperature, acidified with a mixture (1:1 v/v) of conc. HCl and ice, and extracted with ether (4×150ml.). The ether solution was washed with cold water (50ml.) and dried over $CaSO_4$. The solvent was removed and the residue, consisting of crude ethyl-6-fluorohexylmalonic acid, was decarboxylated by heating in an oil bath at 140–150° for 1·5hr. Distillation and fractionation of the product gave 19·2g. (92%) of the pure acid.

The following were obtained in the same way: *2-ethyl-7-fluoroheptanoic acid* (82%), *2-ethyl-9-fluorononanoic acid* (85%), *7-fluoro-2-n-propylheptanoic acid* (83%), *8-fluoro-2-n-propyloctanoic acid* (89%), *9-fluoro-2-n-propylnonanoic acid* (73%), *7-fluoro-2-isopropylheptanoic acid* (70%), *8-fluoro-2-isopropyloctanoic acid* (70%) and *9-fluoro-2-isopropylnonanoic acid* (78%).

β-Alkyl-ω-fluoroalkanoic acids

α-Alkyl-ω-fluoroalkanoyl chlorides. *2-Ethyl-8-fluoro-octanoyl chloride* was prepared as follows. 2-Ethyl-8-fluoro-octanoic acid (25·0g., 0·132mole) was added dropwise to gently refluxing thionyl chloride (21·5g., 0·180mole) at such a rate that the reaction remained under control (about 30min.). The solution was then heated under reflux for a further 2·5hr. and distilled without further treatment. The yield of the acid chloride, b.p. 120°/14mm. Hg, was 25·6g. (94%).

The following were obtained in the same way (yield and b.p. are shown in parentheses): *2-ethyl-7-fluoroheptanoyl chloride* (85%; 105°/12mm. Hg), *7-fluoro-2-n-propylheptanoyl chloride* (82%; 113°/14mm. Hg), *8-fluoro-2-n-propyloctanoyl chloride* (87%; 129°/14mm. Hg), *7-fluoro-2-isopropylheptanoyl chloride* (88%; 126°/14mm. Hg) and *8-fluoro-2-isopropyloctanoyl chloride* (69%; 130°/16mm. Hg).

β-Alkyl-ω-fluoroalkanoic acids. *3-Ethyl-8-fluoro-octanoic acid* was prepared as follows. A solution of 2-ethyl-7-fluoroheptanoyl chloride (14·0g., 0·072mole) in anhydrous ether (80ml.) was added over 15–20min. to a stirred solution of diazomethane (6·0g., 0·143mole) in ether (450ml.) at 0–5°. The solution was stirred for a further 2hr. at that temperature, and was left overnight at room temperature. The solvent was then removed in a rotary evaporator at 20–25°. The yellow residue of crude diazoketone was dissolved in dioxan (250ml.) and was added dropwise to a stirred mixture of freshly precipitated Ag_2O (4g.), Na_2CO_3 (10g.), $Na_2S_2O_3$ (6g.) and water (400ml.) at 70°. This mixture was stirred for 2hr. at 65–70° with intermittent addition of further quantities of Ag_2O to maintain a slight excess at all times. The mixture was heated for 10hr. at 90°, filtered, acidified with dilute HNO_3 and extracted with ether. The ether solution was separated into neutral and acidic fractions with $NaHCO_3$ solution and the acidic fraction (in ether) was dried over $MgSO_4$ and distilled to give the crude acid. Fractionation gave 6·0g. (44%) of the pure product.

3-Ethyl-9-fluorononanoic acid (36%) was obtained in the same way.

9-Fluoro-3-n-propylnonanoic acid was prepared as follows. 8-Fluoro-2-*n*-propyloctanoyl chloride (19·0g., 0·085mole) in anhydrous ether (100ml.) and diazomethane (8·0g., 0·19mole) in anhydrous ether (600ml.) gave the crude diazoketone as in the previous reaction. This was dissolved in dioxan (137ml.) and added to a solution of $AgNO_3$ (1·0g.) in water (15ml.) and aq. NH_3 (sp.gr. 0·88) (29ml.). The resultant liquid was refluxed gently on a water bath for 12hr. The black residue was filtered off; the solution was then diluted with twice its volume of ice–water and extracted with ether (4×100ml.). After removal of the solvent, the resultant crude 9-fluoro-3-*n*-propylnonanoamide was hydrolysed by being heated with a solution of NaOH (40g.) in water (200ml.) and ethanol (50ml.) on a steam bath until a clear solution was obtained and no more NH_3 was evolved. The solution was cooled, acidified with a mixture (1:1, v/v) of conc. HCl and ice, and extracted with ether (4×100ml.). The ether solution was dried over $MgSO_4$, distilled and fractionated to give 10·5g. (57%) of the pure acid.

The following were prepared in the same way: *8-fluoro-3-n-propyloctanoic acid* (37%), *8-fluoro-3-isopropyloctanoic acid* (37%) and *9-fluoro-3-isopropylnonanoic acid* (40%).

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Studies on Metabolism of Vitamin A

THE EFFECT OF THE STAGE OF VITAMIN A DEFICIENCY ON SULPHATE ACTIVATION IN RAT LIVER

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1. The synthesis of 'active sulphate' in rat liver was studied at various stages of vitamin A deficiency, with the corresponding pair-fed controls. 2. The activity was significantly decreased even at the onset of the deficiency, and at the acute stage there was further loss. 3. Only at the earlier stages of the deficiency was the addition of retinol, *in vitro*, fully effective in restoring the lost activity; retinoic acid was partially active. No such restoration was possible at the acute deficiency stage.

The possibility of the existence in animals of a relationship between sulphate metabolism and vitamin A has been suggested by various observations made by several workers. Thus, with tissue-culture experiments, Fell, Mellanby & Pelc (1956) demonstrated that the inclusion of excess of vitamin A in the media causes the production of soluble sulphate. In experiments with whole animals, Dziewiatkowski (1954) showed higher concentrations of serum inorganic sulphate in vitamin A-deficient rats, and Balakhovski & Kuznetsova (1958) reported increased urinary excretion of inorganic sulphate.

However, subsequent work at the enzyme level failed to lead to complete agreement on the relationship between vitamin A and sulphate metabolism. Thus Wolf & Varandani (1960) at first showed a decreased uptake of [^{35}S]sulphate by the mucopolysaccharides of the colon homogenates of vitamin A-deficient rats and suggested that this could be due to a lowered synthesis of 'active sulphate' (PAPS†). Later, Varandani, Wolf & Johnson (1960) demonstrated a decrease in the synthesis of PAPS in the colon homogenates of vitamin A-deficient rats that could be restored by the addition of retinol *in vitro*. Sundaresan & Wolf (1963) again claimed that the synthesis of PAPS is depressed in vitamin A-deficient rat tissues, but they failed to restore the lost activity by the addition of retinol or retinoic acid *in vitro*. Instead, they reported that the addition of a metabolite derived from retinoic acid is effective. This was followed by a preliminary announcement by Sundaresan, Elford & Wolf (1964) claiming the isolation of an enzyme-bound metabolite of vitamin A that is apparently functional in the reactivation of ATP sulphurylase. These observations thus appeared to disagree with the previous findings by Wolf and his co-workers. Simultaneously with those of Sundaresan & Wolf (1963) almost similar claims were made by Rogers, Chang & Johnson (1963) also. In the meantime, Pasternak, Humphries & Pirie (1963) reported that vitamin A deficiency does not affect the synthesis of PAPS in the cornea and colonic mucosa of rats and rabbits. At about the same time we showed that the synthesis of PAPS is depressed in the liver of vitamin A-deficient rats and that the addition of retinol *in vitro* is fully effective in restoring the lost activity, retinoic acid being partially active (Subba Rao & Ganguly, 1964).

Thus, except for Pasternak *et al.* (1963), there has been general agreement that PAPS synthesis is decreased in vitamin A deficiency and the point of disagreement has been on the ability of retinol to restore the lost activity. We have therefore reinvestigated this question and show below, in confirmation of our previous results, that the synthesis of PAPS is decreased in the liver of vitamin A-deficient rats and that it can be reactivated by the addition of vitamin A derivatives *in vitro*; it is also shown below that such reactivation is possible only at the milder stages of the deficiency.

MATERIALS AND METHODS

The sources of the various materials used, the preparation of the particle-free rat-liver supernatant that was used as the source of the enzymes synthesizing PAPS, and the methods of isolation and assay of PAPS were as described by Subba Rao & Ganguly (1964).

The vitamin A-deficient rats were obtained as described

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† Abbreviation: PAPS, adenosine 3′-phosphate 5′-sulphatophosphate.

by Malathi, Subba Rao, Seshadri Sastry & Ganguly (1963). The period of the deficiency was arbitrarily divided into three stages as follows: (i) The weight-plateau stage, which signified the period when the rats maintained a constant weight for 3–4 days. (ii) The stage of mild deficiency, where the animals started losing weight. It immediately followed the weight-plateau stage and lasted for 4–6 days, during which time the animals lost 4–8g. (iii) The stage of severe deficiency, which followed the mildly deficient stage; during this period the rats rapidly lost weight and were highly infected. At all the three stages of the deficiency the enzymic activities of the deficient rats were compared with those of corresponding pair-fed controls.

We have found that pure retinol is unstable both in the crystalline state and in solution, even in the presence of antioxidants. Therefore, for the additions *in vitro*, instead of using synthetic retinol as supplied by the manufacturers we always freshly prepared it enzymically from synthetic retinyl acetate. A sample of retinyl acetate was purified by chromatography on an alumina column by the method of Thompson, Ganguly & Kon (1949) and then hydrolysed by the procedure of Murthy, Mahadevan, Seshadri Sastry & Ganguly (1961), with acetone-dried preparations of rat pancreas as the source of vitamin A ester hydrolase. The alcohol formed was separated from the ester by column chromatography, after which its spectrum, measured in a Beckman model DU spectrophotometer, was always that characteristic of retinol.

For establishing the identity of the PAPS synthesized, the criteria, e.g. its total hydrolysis by 0·1 N-HCl at 37° for 30 min. and its ability to donate about 80% of the sulphate group to *p*-nitrophenol enzymically, were employed, as described by Subba Rao & Ganguly (1964). In addition to these, the compound formed had an absorption maximum at 260 mμ, and when subjected to electrophoresis it moved 20 cm., whereas inorganic sulphate moved 28 cm., which is in agreement with Varandani *et al.* (1960).

RESULTS AND DISCUSSION

The results of the experiments where the synthesis of PAPS was followed at the three stages of the deficiency (Table 1) show that in rat liver it is affected by vitamin A deficiency. However, the loss of the activity, and the ability of added retinol or retinoic acid to restore it, seem to depend on the stage of the deficiency. Thus, though there was some loss at the first two stages (weight-plateau and mildly deficient), which could fully be restored by retinol and partially by the acid, further loss took

Table 1. *Effect of the stage of vitamin A deficiency on the synthesis of* [^{35}S]*PAPS*

The reaction mixtures and the assay of enzymic activities were as described by Subba Rao & Ganguly (1964). The given amounts of retinol or retinoic acid were added to the reaction mixture. All values are averages from three rats; the ranges are given in parentheses.

Status of the rats		Addition	[^{35}S]PAPS formed (counts/min./mg. of protein/hr.)
Weight-plateau stage	Pair-fed	None	37720 (36320–39600)
	Deficient	None	27600 (26900–28430)
	Deficient	5 μg. of retinol in 0·01 ml. of 1% ethanol	36830 (35810–38600)
	Deficient	5 μg. of retinoic acid in 0·01 ml. of phosphate buffer, pH 10·0 (0·2 M)	32670 (31260–34150)
Mildly deficient	Pair-fed	None	33270 (32100–34650)
	Deficient	None	20870 (19000–23600)
	Deficient	5 μg. of retinol in 0·01 ml. of 1% ethanol	32200 (31000–34440)
	Deficient	5 μg. of retinoic acid in 0·01 ml. of phosphate buffer, pH 10·0 (0·2 M)	23930 (22600–25000)
Severely deficient	Pair-fed	None	31330 (30700–32050)
	Deficient	None	16530 (14830–18000)
	Deficient	5 μg. of retinol in 0·01 ml. of 1% ethanol	17060 (14800–20080)
	Deficient	5 μg. of retinoic acid in 0·01 ml. of phosphate buffer, pH 10·0 (0·2 M)	16910 (14600–20000)

place at the severely deficient stage, which could not be reactivated to any extent by the vitamin A derivatives.

There could be several sources of error leading to the conflicting claims, e.g. the identification of the product. That the compound isolated in the present work is PAPS was established by several criteria (cf. the Materials and Methods section). Moreover, since retinol is rather unstable, we have taken extreme care to make sure that the compound used for the additions *in vitro* is pure retinol, and for this purpose we always prepared it freshly from purified retinyl acetate. We have even avoided saponification with ethanolic potassium hydroxide, because peroxides might cause some destruction or structural modification of retinol, and have instead used a much milder method, namely enzymic hydrolysis.

As indicated in the introduction, though there has been general agreement that ATP-sulphurylase activity is decreased during vitamin A deficiency, the point of disagreement was on the ability of retinol to restore *in vitro* the lost activity. However, much of this information (Sundaresan & Wolf, 1963; Sundaresan *et al.* 1964; Rogers *et al.* 1963) has appeared in the form of abstracts, and therefore, in the absence of experimental details, it is difficult for us objectively to discuss these apparently conflicting reports. Nevertheless, in spite of these limitations our present results can offer some explanation. Thus if in their earlier work these workers had used rats that were mildly deficient, whereas in their later experiments their animals were severely deficient, their failure to reactivate with the added retinol in the later trials would agree with our present results, and therefore the claims would cease to be contradictory. Further, if any metabolite of retinol were to participate in this reaction, at the earlier stages of the deficiency the enzyme system responsible for the formation of such a metabolite could have been still active, whereas at the acute stage it might have been totally lost, leading to no formation of the hypothetical metabolite. Similarly, the reported failure to obtain any reactivation by adding retinoic acid or retinol to the purified ATP sulphurylase (Sundaresan & Wolf, 1963) can also be explained on the basis that the enzyme(s) that converts these compounds into the metabolite was probably removed during the process of purification. The present data as well as the present interpretations have been discussed by Subba Rao (1964).

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Effect of a Cholesterol-Biosynthesis Inhibitor on the Fatty Acid Composition of Phospholipids in the Serum and Tissues of Rats

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1. Changes produced by a cholesterol-biosynthesis inhibitor, *trans*-1,4-bis-(2-chlorobenzylaminomethyl)cyclohexane dihydrochloride (AY–9944), in the total fatty acids in the liver and brain, and in phospholipids in the serum, liver, heart, brain and lungs from male rats, have been studied. 2. Treatment with AY–9944 produced the following changes in the fatty acid composition: (*a*) a marked decrease in the percentage of linoleic acid and an increase in oleic acid in the total fatty acids in the liver; (*b*) in the serum, an overall decrease in the percentage of linoleic acid in neutral lipids and phospholipids; (*c*) an increased content of linoleic acid in the β-acyl chain of phosphatidylcholines in the liver and in sphingomyelins in the brain and lungs; (*d*) an increased content of palmitic acid and oleic acid in the β-acyl chain of phosphatidylcholine in the liver, heart and lungs; (*e*) an increased content of phosphatidylcholines and sphingomyelins, together with an increased percentage of saturated fatty acids in these phosphatides in the lungs. 3. Changes in the phosphatides and the production of foam cells in the lungs suggest that AY–9944 may be of use in the study of the alveolar membrane.

Stimulation of fatty acid biosynthesis and concomitant depression of cholesterol biosynthesis have been reported in rat liver (Layne, Rudolph & Bessman, 1960; Foster & Bloom, 1963). *trans*-1,4-Bis-(2-chlorobenzylaminomethyl)cyclohexane dihydrochloride (AY–9944) (Humber, 1964) given orally has been reported to inhibit the biosynthesis of cholesterol (Dvornik, Kraml, Dubuc, Givner & Gaudry, 1963) by interfering with the enzymic conversion of 7-dehydrocholesterol into cholesterol (Kraml, Bagli & Dvornik, 1964). The agent increased the incorporation of labelled acetate into fatty acids (Givner & Dvornik, 1965) and affected the incorporation of labelled acyl and nitrogen base moieties into phospholipids (Hill, 1964).

To investigate the effect of AY–9944 on phospholipids, the changes in the total fatty acid composition of the liver and brain, and of phosphatidylcholine and sphingomyelins from the liver, heart, lung and brain of rats, were determined. To determine whether the availability of cholesterol in the tissues affected the changes produced by AY–9944, rats were fed on a fat-supplemented diet known to elevate the cholesterol content in the liver (Swell, Boiter, Field & Treadwell, 1953).

EXPERIMENTAL

Male albino rats, weighing about 100g., were fed on Purina chow (reference diet) or Purina chow supplemented with 1% of cholesterol, 2·0% of sodium cholate and 7·5% of butterfat (diet A). Treatment consisted of 4·6mg. of AY–9944/kg. body wt. given orally per day. The duration of feeding and treatment is given in each Table.

Serum was extracted with ethanol–ether (3:1, v/v) and the extract evaporated under N_2. The residue was taken up in light petroleum (b.p. 30–40°) and the neutral lipids and phospholipids were separated on silicic acid columns as described by Horning, Williams & Horning (1960). The phospholipids were rechromatographed on silicic acid as described by Newman, Liu & Zilversmit (1960) and the fractions containing phosphatidylserine plus phosphatidylethanolamine, phosphatidylcholine, and lysophosphatidylcholine plus sphingomyelin were collected.

Whole brains and livers from rats fed on either diet were homogenized in ethanol–ether (3:1, v/v). The extract was evaporated under N_2 and the residue hydrolysed with methanolic KOH (1·0ml. of 18N-KOH, 4ml. of methanol and 5 ml. of water) at 70° for 3hr. Phosphatidylcholines and sphingomyelins were isolated by successive silicic acid chromatography (Hill & Rochefort, 1965) from the liver, heart, brain and lungs, and the homogeneity of the isolated phosphatides was checked by thin-layer chromatography (Vogel, Doizaki & Zieve, 1962). Organs were pooled to obtain samples adequate for the fatty acid analysis of the isolated phosphatides. The β-fatty acids of phosphatidylcholines were isolated as described by Hill & Rochefort (1965).

The sphingomyelins were hydrolysed with methanolic HCl (1·0ml. of 6N-HCl, 4ml. of methanol and 5ml. of water) at 70° for 3hr. (Sweeley & Moscatelli, 1959–60). The fatty acids were extracted twice with ether, the solvent was evaporated under N_2 and the residue was taken up in pentane. Methylation was accomplished with diazomethane (Schlenk & Gellerman, 1960). The methyl esters were

Table 1. *Percentage composition of the fatty acids of serum neutral lipids and phospholipids in rats given AY–9944*

Serum (12 ml.) was obtained from four male rats fed on the reference diet and given 4·6 mg. of AY–9944/kg./day orally for 28 days. Neutral lipids and phospholipids were separated as described in the text. The fatty acids were identified by their retention times relative to palmitic acid on butane-1,4-diol succinate polyester at 210°. Fatty acid relative retention times for 18:2–20:4 acids have been combined. In all Tables (and in the text) the first figure of the fatty acid designation represents the no. of carbon atoms/mol. and the second figure the no. of double bonds/mol. The percentage composition is calculated as: 100 × peak area of fatty acid/total peak area. — indicates that the amount of the fatty acid present was less than 0·5%.

		Percentage composition of fatty acids							
		Neutral lipids		Phosphatidylserines and phosphatidylethanolamines		Phosphatidylcholines		Lysophosphatidylcholines and sphingomyelins	
Relative retention time	Fatty acid	Reference diet	Reference diet +AY–9944	Reference diet	Reference diet +AY–9944	Reference diet	Reference diet +AY–9944	Reference diet	Reference diet +AY–9944
< 0·86	12:0 to 16:0	4·2	7·0	4·3	12·9	0·7	2·6	5·0	0·8
1·00	16:0	21·4	33·9	25·2	22·1	25·2	27·7	27·0	83·9
1·10	16:1	1·9	2·9	2·0	2·0	1·6	1·6	1·4	0·5
1·31	17:0	0·8	1·7	10·2	9·9	1·1	1·9	3·2	0·6
1·70	18:0	14·0	8·2	10·3	6·4	14·7	14·9	19·2	3·4
1·86	18:1	21·9	19·1	23·1	22·4	23·7	25·1	23·9	5·1
2·21	18:2	21·6	10·2	17·5	10·5	22·8	19·0	13·1	2·6
2·60–4·49	18:2 to 20:4	4·4	2·0	3·4	2·0	—	0·8	3·1	2·5
4·94	20:4	4·9	10·6	3·2	2·6	3·9	5·9	—	—
5·40–11·94	above 20:4	2·7	0·8	—	6·4	0·9	—	—	1·4

separated on butane-1,4-diol succinate polyester coated on Chromosorb W at 210° (Perkin–Elmer gas chromatograph model 800) or at 185° (Pye Argon chromatograph). Standard mixtures of methyl esters were kindly supplied by the National Institutes of Health, Bethesda, Md., U.S.A., and pure standards were purchased from the Hormel Institute, Austin, Minn., U.S.A.

RESULTS

Serum lipids. In the neutral lipids, treatment with AY–9944 increased the percentage of short-chain fatty acids (between 12:0 and 16:0) and of palmitic acid and arachidonic acid, and decreased that of stearic acid and linoleic acid (Table 1).

In the serum of rats treated with AY–9944, in the phosphatidylserine and phosphatidylethanolamine fraction the percentage of short-chain fatty acids was increased from 4 to 13%, and those of stearic acid and linoleic acid were decreased from 10 to 6% and from 17·5 to 10·5% respectively. In the fraction containing lysophosphatidylcholines and sphingomyelins, treatment increased the palmitic acid content from 27 to 84%. Little change occurred in the fatty acid composition of serum phosphatidylcholines.

Tissue lipids. The fatty acid composition of the liver and brain lipids of rats fed on either reference diet or diet A as determined by gas–liquid chromatography is shown in Tables 2 and 3. In the liver of rats given diet A, the oleic acid content increased from 7·0 to 33%, whereas stearic acid decreased from 31 to 12% and arachidonic acid from 20·5 to 3%. Little change in the fatty acid composition of the brain lipids occurred in rats fed on diet A.

On treatment with AY–9944, the content of palmitic acid and oleic acid increased, whereas the content of stearic acid, linoleic acid and arachidonic acid decreased to different extents in the liver of rats fed on the reference diet. In rats fed on diet A, AY–9944 decreased the content of linoleic acid and increased the content of oleic acid in the liver. The content of palmitic acid and stearic acid decreased on treatment in the brain.

Phosphatidylcholines. The fatty acid composition of the β-acyl chain of phosphatidylcholines in the brain, heart, liver and lungs is shown in Table 4. AY–9944 increased the percentage of palmitic acid plus stearic acid in the lungs from 37 to 50% and decreased the percentage in the brain from 43·6 to 35%. On treatment with AY–9944, the content of palmitic acid and oleic acid increased slightly in the liver and markedly in the heart and lungs. Treatment decreased the content of the short-chain fatty acids in the heart and produced an overall decrease in the fatty acid content in the brain.

Table 2. *Percentage composition of the fatty acids of liver lipids of rats given AY–9944*

Male hooded rats were fed on the reference diet for 38 days and treated rats were given 4·6 mg. of AY–9944/kg./day from day 11 to day 38. The fatty acid composition was determined on the total lipids from eight pooled livers. Samples were analysed on butane-1,4-diol succinate polyester (10% on Chromosorb W) at 185° (Pye Argon chromatograph). The percentage composition is calculated as indicated in Table 1. — indicates that the amount of the fatty acid present was less than 0·5%.

	Percentage composition of fatty acids			
Fatty acid	Reference diet	Reference diet +AY–9944	Diet A	Diet A +AY–9944
16:0	17·9	24·1	21·0	24·8
16:1	—	1·4	—	3·0
18:0	30·8	24·4	12·1	10·4
18:1	6·6	16·7	33·0	51·5
18:2	21·1	10·7	16·5	6·1
20:4	20·5	16·1	3·1	2·8

Table 3. *Percentage composition of the fatty acids of brain lipids in rats given AY–9944*

Experimental details are as given in Table 2. The percentage composition is calculated as indicated in Table 1. — indicates that the amount of the fatty acid present was less than 0·5%.

	Percentage composition of fatty acids			
Fatty acid	Reference diet	Reference diet +AY–9944	Diet A	Diet A +AY–9944
15:0	3·1	—	0·6	—
16:0	33·3	22·5	31·1	24·7
16:1	—	0·7	0·6	1·0
18:0	19·3	15·1	21·0	15·8
18:1	28·1	32·4	31·5	31·6
18:2	2·5	—	0·7	—
20:0	—	4·1	—	4·1
20:4	8·7	8·2	5·4	10·3

The relative proportions of the fatty acids between 18:2 and 20:4 (relative retention 2·38 to 4·38) varied with the tissue. On treatment, the composition and percentage of these fatty acids were altered in the brain and lungs. The content of long-chain fatty acids (above 20:4) decreased on treatment in the lungs.

Sphingomyelins. The amount of saturated fatty acids (16:0 and 18:0) in the liver, the brain and the heart varied from 43·5 to 49%, but comprised only 21% of the lung sphingomyelin fatty acids. The total content of sphingomyelin fatty acids was increased in the brain and lung on treatment (Table 5).

In the brain, the content of palmitic acid, palmitoleic acid and oleic acid increased, whereas that of linoleic acid decreased on treatment. Similar changes occurred in the lungs, except that the percentage of oleic acid decreased. In both the lungs and brain, the percentage of long-chain fatty acids (above 20:4), including the 22:1, 24:0, 24:1 and 22:6 acids, decreased markedly on treatment.

In the heart, the change on treatment in the composition of the fatty acids appeared as a decrease in linoleic acid and an increase in arachidonic acid.

DISCUSSION

The physiological relationship between cholesterol and fatty acid biosynthesis is little understood. Wood & Migicovsky (1957) have suggested a correlation between inhibition of cholesterol biosynthesis and elongation of the carbon chain in saturated fatty acids. Citrate has been reported to stimulate fatty acid biosynthesis (Srere, 1965), while depressing cholesterol biosynthesis (Foster & Bloom, 1963). Catravas (1963) has isolated a protein–carbohydrate complex (lipogenin) that stimulated biosynthesis of both fatty acids and cholesterol.

Table 4. *Percentage composition of the β-acyl fatty acids of tissue phosphatidylcholines of rats given AY–9944*

The β-fatty acids of phosphatidylcholines were isolated by successive silicic acid chromatography, followed by hydrolysis with *Crotalus adamenteus* venom and subsequent isolation of the β-fatty acids as described by Hill & Rochefort (1965). The fatty acids were identified by their retention times relative to palmitic acid on butane-1,4-diol succinate polyester at 210°. The percentage composition is calculated as indicated in Table 1. — indicates that the amount of the fatty acid present was less than 0·5%.

		Percentage composition of fatty acids							
		Liver		Heart		Brain		Lung	
Relative retention time	Fatty acid	Reference diet	Reference diet +AY–9944	Reference diet	Reference diet +AY–9944	Reference diet	Reference diet +AY–9944	Reference diet	Reference diet +AY–9944
	below 16:0	2·8	2·2	35·8	11·8	3·3	3·2	7·6	7·9
1·00	16:0	17·2	26·4	10·2	14·3	11·4	10·3	14·4	20·9
1·11	16:1	1·9	1·9	2·1	1·8	—	—	—	—
1·26–1·59	16:1 to 18:0	1·7	0·7	1·8	1·0	0·6	2·5	1·9	2·4
1·70	18:0	12·1	10·8	32·1	27·6	32·2	24·4	20·3	28·7
1·86	18:1	26·0	28·9	13·2	14·2	37·3	34·1	11·8	19·4
2·10		—	—	—	—	1·3	1·5	—	3·4
2·20	18:2	17·0	18·0	5·0	—	—	—	1·7	1·1
2·38–2·88*		1·9	1·2	—	—	—	1·8	—	—
3·11		0·7	1·0	—	—	0·6	4·8	1·3	0·8
3·39		—	—	—	—	7·3	6·7	1·2	1·2
3·69–3·96†		0·6	—	—	29·5	0·6	0·9	1·3	3·0
4·20		7·7	1·3	—	—	0·8	1·2	19·2	—
4·38		—	—	—	—	—	4·4	—	5·8
4·85	20:4	7·5	5·1	—	—	2·0	2·8	—	—
5·10–5·63	above 20:4	2·0	2·0	—	—	—	—	—	—
5·10–6·80		—	—	—	—	1·6	1·0	—	—
5·10–10·80		—	—	—		—	—	12·4	4·3
Wt. of β-fatty acids (mg.)		202	233	6	12	43	28	16	27
Wet wt. of tissue (g.)		44·1	45·1	3·8	3·2	6·0	4·9	5·5	5·1
Wt. of β-fatty acids/g. wet wt. of tissue		4·58	5·17	1·58	3·75	7·17	5·71	2·91	5·29

* Contains three fatty acid peaks.
† Contains two fatty acid peaks.

Alternatively, it has been suggested that biosynthesis of fatty acids (Bortz & Lynen, 1963) and cholesterol (Siperstein & Bhattathirty, 1963) may be controlled by an end-product feedback mechanism, although the interdependence of the feedback is unknown.

In the present study, the addition of cholesterol together with cholate and butterfat to the diet after 28 days caused a decrease in the percentage of linoleic acid and arachidonic acid in the liver lipids, but caused little change in the brain lipids, whose fatty acid composition was comparable with that reported by Biran & Bartley (1961). The addition of cholesterol to the diet has been reported to alter phospholipid polyunsaturated fatty acids in the liver (Morton & Horner, 1961).

In the liver of rats fed on the reference diet, treatment with AY–9944 produced a marked decrease in the percentage of linoleic acid and an increase in oleic acid, whereas in the brain the percentage of palmitic acid and stearic acid decreased. In rats fed on the supplemented diet, the changes in the fatty acid composition of the liver on treatment were less marked, except that the oleic acid increased to approx. 50% of the total fatty acids.

It has been postulated that the rate of turnover of phospholipids may be related to their role in metabolism (Collins, 1963; Redman & Hokin, 1959), which may vary with their fatty acid composition (Wurster & Copenhaver, 1965). Stoffel & Ach (1964) have reported that fatty acids biosynthesized in different tissues are characteristic of the tissue phosphatides, and Lands & Hart (1965) found different patterns of acyltransferases in different tissues.

Table 5. *Percentage composition of the fatty acids of tissue sphingomyelins of rats given AY–9944*

Experimental conditions are given in the text. Fatty acids with relative retentions 2·80–4·38 and 5·34–11·49 have been combined for clarity. The percentage composition is calculated as indicated in Table 1. — indicates that the amount of the fatty acid present was less than 0·5%.

		Percentage composition of fatty acids							
		Liver		Heart		Brain		Lung	
Relative retention time	Fatty acid	Reference diet	Reference diet +AY–9944	Reference diet	Reference diet +AY–9944	Reference diet	Reference diet +AY–9944	Reference diet	Reference diet +AY–9944
0·37–0·74	below 16:0	—	—	—	—	1·4	—	1·7	8·6
1·00	16:0	19·9	14·3	22·3	22·3	15·2	33·3	13·4	62·9
1·13	16:1	0·5	0·7	—	—	0·6	3·1	24·7	7·1
1·74	18:0	23·6	23·8	26·3	26·0	32·2	16·0	6·5	4·1
1·92	18:1	10·0	12·3	13·5	13·3	18·6	27·0	16·7	7·8
2·28	18:2	25·5	25·8	31·1	22·4	1·6	—	9·3	5·4
2·80–4·38	18:2 to 20:4	1·7	1·8	0·8	0·5	1·5	1·3	1·9	—
4·80	20:4	14·2	16·6	1·7	12·7	15·1	9·9	18·4	2·9
5·34–11·49	above 20:4	3·4	3·4	3·3	2·0	13·9	7·7	7·1	—
Wt. of fatty acids in sphingomyelin (mg.).....		36	36	21	16	2	4	3	21
Wet wt. of tissue (g.).....		24·1	45·1	3·8	3·2	6·0	4·9	5·5	5·1
Wt. of sphingomyelin fatty acids/g. wet wt. of tissue (mg.)		0·82	0·80	5·53	5·00	0·33	0·82	0·55	4·12

Further, Lands & Hart (1965) have indicated that certain fatty acids in phosphatidylcholines may affect the rate and specificity of the acyltransferases, and Leat (1964) suggested that the function of aminophosphatides, e.g. phosphatidylethanolamines, was dependent on their arachidonic acid content. Treatment with AY–9944 produced a marked increase in the content of phosphatidylcholines in the heart and lungs and a slight increase in the liver. This increase may reflect an increased synthesis of phosphatidylcholines in these tissues.

Though phosphatidylcholines may be synthesized via a similar pathway in the liver and brain (Strickland, Subrahmanyam, Pritchard, Thompson & Rossiter, 1963), the treatment produced opposite effects on the β-fatty acid content of phosphatidylcholines in the brain and liver. Specific changes in different tissues, such as the decrease in content of short-chain fatty acids (between 12:0 and 16:0) in the heart and in the long-chain fatty acids (above 20:4) in the lungs, further suggested that AY–9944 either affected the fatty acid biosynthesis or the acyltransferases or both in the tissues differently. However, the increased content of palmitic acid and oleic acid in sphingomyelin in the brain and lungs suggested a similar effect on the fatty acid metabolism in these tissues.

Allman (1964) has reported that the amount of fatty acids synthesized in a tissue, measured either by incorporation of [2-^{14}C]acetate or oxidation of NADPH, was proportional to the linoleic acid content in the tissues. The increased content of linoleic acid in brain and lung sphingomyelin and in the β-acyl chain of phosphatidylcholines in the liver together with the increase in the fatty acid content in these tissues suggest that AY–9944 may have increased fatty acid biosynthesis.

It is noteworthy that the percentage of linoleic acid decreased in the neutral lipids and phosphatides and the percentage of palmitic acid increased in neutral lipids, lysophosphatidylcholines and sphingomyelins in the serum, since similar changes in linoleic acid and palmitic acid were found in the fatty acid composition of the liver on treatment with AY–9944.

Rat lungs have been reported to incorporate long-chain fatty acids into phospholipids (Felts, 1962) and [^{14}C]acetate into mitochondrial lipids (Tombropoulus, 1964). Klaus, Reiss, Tooley, Piel & Clements (1962) have shown that mitochondria in alveolar epithelial cells secrete a surface-active lipid–protein complex, probably dipalmitoylphosphatidylcholine (Brown, 1964; Klaus, Clements & Havel, 1961), which stabilizes the alveolar radius (Clements, 1962). Further, Schaefer, Jordan & Thelin (1965) correlated changes in the surface-active complex and lamellar bodies in granular pneumocytes with disruption of the hyaline membrane.

Treatment with AY–9944 has been reported to

increase the incorporation of labelled palmitic acid and choline into lung sphingomyelins (Hill & Rochefort, 1965). In rat lungs phosphatidylcholines and sphingomyelins comprise 34 and 26% respectively of the total phospholipids (Marinetti, Erbland & Stotz, 1958).

The decreased percentage of long-chain fatty acids in lung phosphatidylcholines and sphingomyelins and the marked increase in saturated fatty acids, mainly palmitic acid in the latter, on treatment may affect the function of the phosphatides (Farquhar, 1965). The increased content of lung phosphatidylcholines and sphingomyelins is of added interest since AY-9944 has been reported to produce foam cells in rat lungs (Greselin, 1965).

Evidence indicates that AY-9944, an inhibitor of cholesterol biosynthesis, affected the fatty acid composition of the β-acyl chain of phosphatidylcholines and sphingomyelins. Further, the agent may be of use in the investigation of the phospholipid metabolism of the alveolar membrane.

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Effects of Cystamine and Cysteamine on the Adenosine-Triphosphatase Activity and Oxidative Phosphorylation of Rat-Liver Mitochondria

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1. Cystamine (2,2′-diaminodiethyl disulphide) caused an unmasking of mitochondrial adenosine triphosphatase and a leakage of Mg^{2+} from the mitochondria, and decreased the stimulation of adenosine triphosphatase by 2,4-dinitrophenol. When Mg^{2+} was added, cystamine potentiated the activation of adenosine triphosphatase by 2,4-dinitrophenol. 2. Cystamine was without effect on the adenosine triphosphatase of disrupted mitochondria. 3. Cystamine was moderately potent as an uncoupling agent and as an inhibitor of the $[^{32}P]P_i$–ATP exchange reaction. 4. Cysteamine (2-aminoethanethiol) was without the above effects, when special precautions were taken to counteract its autoxidation. 5. The effects of cystamine should probably be ascribed to its disulphide group, since the diamine cadaverine protected slightly against the loss of Mg^{2+} and the decrease of 2,4-dinitrophenol-stimulated adenosine-triphosphatase activity caused by aging of the mitochondria. It is suggested that cystamine acts by a breakdown of mitochondrial permeability barriers.

Disulphides and thiols of the cysteine–cystamine group are well-known protectors against the effects of ionizing radiation. Further information on the biochemical effects of these compounds has recently been requested (Eldjarn, 1962; Bacq & Alexander, 1964), as such knowledge might contribute to a better understanding of their mode of action.

Some observations on their effects on cellular and subcellular functions have already been made. Thus disulphides of low molecular weight inhibit cellular respiration (Ciccarone & Milani, 1964) and mitochondrial oxygen consumption (Eldjarn, 1962; Lelievre, 1963; Eldjarn & Bremer, 1963). Cystamine and related disulphides apparently interfere with the binding of nicotinamide nucleotides in the mitochondria (Skrede, Bremer & Eldjarn, 1965), and block all NAD-dependent oxidations by isolated mitochondria through a loss of cofactors. Cystamine also uncouples oxidative phosphorylation (van Bekkum & de Groot, 1956; Park, Meriwether, Park, Mudd & Lipmann, 1956) and induces mitochondrial swelling (Neubert & Lehninger, 1962).

Some of the effects of cystamine are probably due to a formation of mixed disulphides between cystamine and the thiol groups of mitochondrial proteins. By this type of reaction cystamine, which is of a low oxidation potential, acts as a specific and mild thiol-blocking agent, combining with highly reactive thiol groups only (Pihl & Eldjarn, 1958; Pihl & Lange, 1962). Further studies on the interaction of cystamine with mitochondria might therefore contribute to the elucidation of the reactivity of mitochondrial thiol groups and their role in mitochondrial functions. Mitochondrial membrane proteins contain substantial amounts of thiol groups (Riley & Lehninger, 1964); the blockage of such groups causes a rapid swelling of the mitochondria (Tapley, 1956; Riley & Lehninger, 1964). It has been suggested that thiol groups have intimate association with oxidative phosphorylation (Kielley, 1961; Fluharty & Sanadi, 1963; Riley & Lehninger, 1964), and that mitochondrial ATPase* has 'inhibitory' thiol groups as well as thiol groups essential for the function of the enzyme (Cooper, 1960).

The present paper deals with the effects of the disulphide cystamine (2,2′-diaminodiethyl disulphide) on oxidative phosphorylation, mitochondrial ATPase, $[^{32}P]P_i$–ATP exchange reaction and the retention of Mg^{2+} by the mitochondria. The effects of the chemically related diamine cadaverine (1,5-diaminopentane) and the corresponding thiol cysteamine (2-aminoethanethiol) were also investigated. The main effect of cystamine appears to be to cause an increase of the permeability of the mitochondrial membrane(s), whereas cadaverine

* Abbreviation: ATPase, adenosine triphosphatase.

and cysteamine are without this effect. The results support the concept that the effects of cystamine are due to its disulphide group.

EXPERIMENTAL

Materials. ATP (disodium salt) was a product of Sigma Chemical Co., St Louis, Mo., U.S.A. Hexokinase was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Cystamine and cysteamine were obtained from Fluka A.-G., Chemische Fabrik, Buchs SG, Switzerland. Cadaverine was a product of British Drug Houses Ltd., Poole, Dorset. [^{32}P]P_i was obtained from the Institute of Atomic Energy, Kjeller, Norway. Naphthalene and dioxan were white-label reagents from Eastman Organic Chemicals, Rochester, N.Y., U.S.A. 2,5-Diphenyloxazole and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene were obtained from Packard Instrument Co. Inc., La Grange, Ill., U.S.A. Sephadex was supplied by AB Pharmacia, Uppsala, Sweden. 2-Aminoethyl hydrogen sulphate was obtained from L. Light and Co. Ltd., Colnbrook, Bucks., and was recrystallized from aqueous ethanol. *N*-Acetyl-DL-homocysteine thiolactone was a product of Schwarz Bioresearch Inc., Mount Vernon, N.Y., U.S.A. All other commercial reagents were products of high purity.

Thiolated Sephadex was prepared by the method of Eldjarn & Jellum (1963). Slight modifications of the method were worked out in co-operation with E. Jellum. The starting material was Sephadex G-25 of medium porosity (65g.) that was rapidly stirred into a chilled solution of NaOH (60g.) and 2-aminoethyl hydrogen sulphate (20g.) in water (156ml.). After a few minutes the swollen material was spread out on a glass plate and dried at 120° overnight. The aminated Sephadex thus formed was repeatedly washed with water until free from alkali. To the total amount of swollen slurry was then added *N*-acetylhomocysteine thiolactone (12g.) in water (800ml.). Under vigorous stirring successive small volumes of M-$AgNO_3$ were added followed by N-NaOH to maintain the pH at 7·5–8·0, until a total amount of 75·5ml. of $AgNO_3$ had been added. It is essential that the pH be checked frequently at this stage of the procedure, since the Ag^+ ions may be reduced by the end groups of the cross-linked dextran if the pH exceeds 9. The suspension was allowed to stand with frequent stirring for 2hr. at room temperature, while the pH was maintained at 8·0 by the frequent addition of small amounts of N-NaOH. The Ag^+ ions were removed with thiourea (140g. in the minimum amount of water) added together with N-HNO_3 (250ml.). The thiolated Sephadex was washed a further 3 times with thiourea (45g.) in 0·01N-HNO_3 and finally several times with water. The swollen suspension was stored at 0–4°, preferably in water, as homocysteine is slowly split off in neutral or alkaline buffers. If dried with ethanol followed by ether and kept in the cold, the material may be stored for months. The number of thiol groups was determined by adding an excess of a neutral solution of tetrathionate to a sample of the thiolated Sephadex and titrating the thiosulphate formed with iodine. The thiolated Sephadex usually contained approx. 0·55–0·45% of thiol groups. Before use the material was treated with cysteine (10mM) to remove trace metals, washed repeatedly with water and resuspended in 0·15M-KCl.

Methods. Rat-liver mitochondria were prepared by the method of Myers & Slater (1957). The mitochondria were washed once and resuspended in 0·25M-sucrose. Disintegration of the mitochondria, when required, was accomplished by treating ice-cold suspensions with ultrasonic vibrations (20000 cyc./sec.) with a Branson Sonifier (model S75) for 6×10sec. at 6A.

ATPase assay. The method used for determining ATPase activity was based on that given by Myers & Slater (1957). All experiments were carried out at pH7·4 in a medium containing tris–HCl buffer (0·05M), EDTA (1mM) and sucrose (0·05M), with or without $MgCl_2$ (5mM) or 2,4-dinitrophenol (0·25 or 0·13mM). The thiol or the disulphide was added as stated in the individual experiments. The incubation volume was made up to 2ml. with KCl (0·07–0·105M). The reaction was started with ATP (2·5 or 5mM) except for the preincubation experiments, where the addition of mitochondria initiated the experimental period. All incubations were performed at 30° and the assay period was always 10min. The reaction was stopped by the addition of 1ml. of 10% (w/v) trichloroacetic acid. The proteins were removed by centrifugation, and P_i was determined in the supernatant fluid by the method of Berenblum & Chain (1938) as modified by Martin & Doty (1949).

Cysteamine autoxidizes rapidly in air at pH7·4. During incubations exceeding 10–15min., a significant fall in the thiol titre was observed. In experiments with cysteamine where the preincubation period and incubation period together exceeded 15min., the autoxidation of the thiol was counteracted by thiolated Sephadex, which is a particulate material functioning as an electron reservoir for the continuous reduction of cystamine formed by the autoxidation of the thiol (Jellum, 1964). The material itself was shown not to affect the ATPase activity, but maintained the thiol titre at the initial level for more than 2hr. The thiol content of the reaction mixture at the end of the incubations was estimated by amperometric silver titration at the rotating platinum electrode (Benesch, Lardy & Benesch, 1955). The modification of the method introduced by Børresen (1963) was used.

Oxidative phosphorylation. The oxygen uptake was measured in a Warburg apparatus. The esterification of phosphate was determined by the disappearance of P_i in the presence of a phosphate-trapping system. The assay medium contained the following. The main chamber contained: potassium phosphate buffer, pH7·4, 30μmoles; $MgCl_2$, 15μmoles; ATP, 2·5μmoles; mitochondria, approx. 9mg. of protein (7mg. in Expt. 3 in Table 5) suspended in 0·15M-sucrose; and (in Expts. 1 and 3 in Table 5) cystamine or cysteamine in the concentrations stated. The volume was made up to a total of 3ml. with 0·15M-KCl. The side arm contained: hexokinase, 1·5mg.; glucose, 100μmoles; succinate, 20μmoles; and (in Expts. 2 and 4 in Table 5) cystamine or dinitrophenol in the concentrations stated. When cysteamine was present, thiolated Sephadex (30mg. dry wt., suspended in 0·15M-KCl) was also added. The contents of the side arm were added after temperature equilibration for 7min. The reaction was run for 10 or 15min. at 30°, and was stopped by tipping in 0·2ml. of 6N-$HClO_4$ from the second side arm. The proteins were spun down, and P_i was determined in the supernatant by the method of Martin & Doty (1949).

[^{32}P]P_i–*ATP exchange reaction.* A slight modification of the method given by Wadkins & Lehninger (1963) was

used. The reaction system contained: ATP, 5 μmoles; tris–HCl buffer, pH 7·4, 50 μmoles; phosphate buffer, 10 μmoles containing approx. 600000 counts/min. of ^{32}P; and mitochondria, 1·2–1·5 mg. of protein. The total volume was 1 ml. Incubations were performed at 30° for 10 min. The reaction was started by the addition of mitochondria and stopped with 1 ml. of 10% trichloroacetic acid. The proteins were removed by centrifugation, and 0·5 ml. of the supernatant fluid was used for the analysis of ^{32}P-labelled ATP. The extraction and washing procedures were undertaken in accordance with the method of Rose & Ochoa (1956). The radioactivity of the sample was determined by counting 1 ml. of the aqueous phase in a Tri-Carb liquid scintillation spectrometer (Packard Instrument Co Inc.), with 15 ml. of scintillator consisting of 12·5% naphthalene, 0·75% 2,5-diphenyloxazole and 0·0375% 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene in dioxan. The extent of incorporation of $[^{32}P]P_i$ into ATP was calculated as described by Wadkins & Lehninger (1963).

Ca^{2+} and Mg^{2+} determinations. In these experiments (indicated in Fig. 3) mitochondria containing approx. 50 mg. of protein were used/incubation tube. The mitochondria were incubated at 30° for 5, 10 or 15 min. in a medium containing: tris–HCl buffer, pH 7·4, 0·10 M; sucrose, 0·01–0·04 M; and the following where indicated: cystamine, 5 mM; cysteamine, 10 mM; cadaverine, 5 mM; dinitrophenol, 0·25 mM. The reaction was stopped by rapid cooling in ice to 0°, and the mitochondria were sedimented by centrifugation at 22000 *g* for 10 min. After decantation the tubes were blotted dry, and the pellet was treated with 1 ml. of 10% $HClO_4$. The proteins were spun down, and the Ca^{2+} and Mg^{2+} content of the neutralized $HClO_4$ extract was determined by photoelectric titration with EDTA (Gjessing, 1959). Mg^{2+} was also determined in the ashes of the $HClO_4$-insoluble material but only insignificant amounts were usually found.

Protein was determined by a biuret method (Goa, 1955).

RESULTS

In the experiments given in Table 1, the ATPase activity of intact mitochondria was low, in accordance with previous observations (Kielley & Kielley, 1951; Myers & Slater, 1957). Only a slight stimulation by Mg^{2+} could be observed. 2,4-Dinitrophenol stimulated the ATPase 10–20-fold when freshly prepared mitochondria were used. Mg^{2+} sometimes caused an additional stimulation of dinitrophenol-stimulated ATPase (Expts. 2 and 3 in Table 1), but in most experiments no such effect was present.

Cystamine, cysteamine or cadaverine at concentrations below 5 mM caused no significant stimulation of ATPase activity in the presence or absence of Mg^{2+} when the preincubation period was less than 5 min. (Table 1). When cystamine was added together with dinitrophenol, the ATPase activity was strongly inhibited as compared with the values with dinitrophenol alone (Table 1). On the other hand, cysteamine or cadaverine did not have an inhibitory action on dinitrophenol-stimulated ATPase.

The inhibition of dinitrophenol-stimulated ATPase induced by cystamine could be completely reversed when Mg^{2+} was also added (Table 1). In fact, the ATPase activity of intact mitochondria in the presence of cystamine, dinitrophenol and Mg^{2+} was higher than the values obtained with dinitrophenol alone. Cysteamine or cadaverine, on the other hand, did not cause any increase of the activity obtained in the presence of dinitrophenol and Mg^{2+} (Table 1).

Table 1. *Effects of cystamine, cysteamine and cadaverine on the ATPase activity of fresh mitochondria*

Freshly prepared mitochondria (approx. 0·5 mg. of protein/tube) were subjected to temperature equilibration for 5 min. in the presence of all additions except ATP. ATP was added at zero time. The following concentrations were used: cystamine, 5 mM; cysteamine, 10 mM; cadaverine, 5 mM; dinitrophenol, 0·25 mM; $MgCl_2$, 5 mM; ATP, 2·5 mM (Expts. 1 and 2) or 5 mM (Expt. 3).

Expt. 1 (with cystamine)		Expt. 2 (with cysteamine)		Expt. 3 (with cadaverine)	
Addition	ATPase activity (μmoles/hr./mg. of protein)	Addition	ATPase activity (μmoles/hr./mg. of protein)	Addition	ATPase activity (μmoles/hr./mg. of protein)
None	0·5	None	1·0	None	3·8
Mg^{2+}	0·8	Mg^{2+}	2·7	Mg^{2+}	6·1
Cystamine	1·5	Cysteamine	0·9	Cadaverine	4·2
Mg^{2+} + cystamine	1·2	Mg^{2+} + cysteamine	1·7	Mg^{2+} + cadaverine	6·2
Dinitrophenol	11·4	Dinitrophenol	9·2	Dinitrophenol	12·8
Dinitrophenol + cystamine	2·2	Dinitrophenol + cysteamine	9·8	Dinitrophenol + cadaverine	12·2
Dinitrophenol + Mg^{2+}	11·6	Dinitrophenol + Mg^{2+}	12·2	Dinitrophenol + Mg^{2+}	17·6
Dinitrophenol + cystamine + Mg^{2+}	15·9	Dinitrophenol + cysteamine + Mg^{2+}	10·5	Dinitrophenol + cadaverine + Mg^{2+}	17·3

The effect of different concentrations of cystamine on the ATPase activity of fresh mitochondria preincubated in the presence of the disulphide for 5min. is shown in Fig. 1. Cystamine alone caused no significant stimulation. When Mg^{2+} was also added, the ATPase activity was moderately increased at a cystamine concentration of 10mM (Fig. 1). Fig. 1 also shows that the inhibition of dinitrophenol-stimulated ATPase by cystamine was enhanced with increasing concentrations of the disulphide. The activation of dinitrophenol-stimulated ATPase by cystamine in the presence of Mg^{2+} was accentuated at higher concentrations.

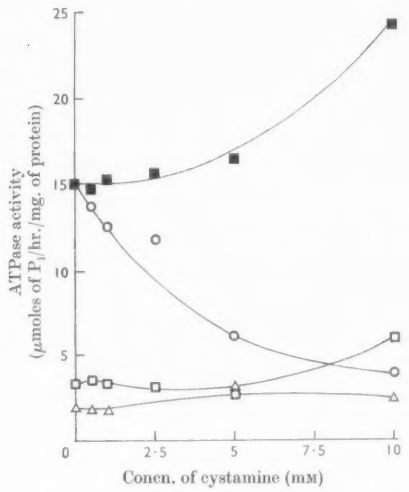

Fig. 1. Effect of cystamine on the ATPase activity of fresh mitochondria. The experimental conditions were the same as those given in Table 1; 10μmoles of ATP were used. Additions: △, none; □, Mg^{2+}; ○, dinitrophenol; ■, Mg^{2+}+dinitrophenol.

When the preincubation period was increased to 10min. at 30°, cystamine caused a complete unmasking of Mg^{2+}-activated ATPase. Cadaverine did not accelerate the unmasking, nor did cysteamine, when its autoxidation was counteracted. My results thus did not confirm the brief report by Åkerfeldt (1963) that cysteamine causes a moderate stimulation of mitochondrial ATPase. In the present study, a moderate inhibition by all three compounds was observed when the Mg^{2+}-activated ATPase of the controls was completely unmasked after 2hr. of aging (Table 2).

It is well known that the ATPase-stimulating effect of dinitrophenol is diminished and finally lost by aging. It has also been shown that mitochondria lose Mg^{2+} during aging in the presence of deoxycholate (Siekevitz, Löw, Ernster & Lindberg, 1958) or P_i (Siekevitz & Potter, 1955*a*). One of the requirements for dinitrophenol-induced stimulation of the ATPase activity of fresh mitochondria is obviously the presence of bound Mg^{2+} in the mitochondria (Cooper, 1958). It has therefore been suggested that the loss of activation by dinitrophenol on aging is due to a release of bound Mg^{2+} from the mitochondria.

Fig. 2(*a*) shows that mitochondria preincubated ('pre-aged') in the presence of dinitrophenol had lost 70% of their dinitrophenol-stimulated ATPase activity after 20min. Cystamine (5mM) increased the loss of dinitrophenol-stimulated ATPase activity significantly, so that the residual activity after 20min. of aging was only 10%. The inactivation of dinitrophenol-stimulated ATPase was not accelerated by cysteamine, and was even delayed by

Table 2. *ATPase activity of mitochondria aged in the presence of cystamine, cysteamine or cadaverine*

Intact mitochondria were incubated at 30° for 10 or 120min. in an iso-osmotic tris–sucrose medium, with or without the addition of cystamine, cysteamine or cadaverine. Then 10ml. of ice-cold 0·25M-sucrose was added, and the mitochondria were spun down at 22000*g* for 10min. and resuspended in 0·25M-sucrose. The assay was then immediately performed as described in the Methods section, with 10μmoles of ATP/tube. The addition of mitochondria (0·4–0·5mg. of protein) started the experiments.

		ATPase activity (μmoles of P_i/mg. of protein/hr.)				
			Agents present during aging			
Duration of aging	Assay conditions	Unaged control	None	Cystamine (5mM)	Cysteamine (10mM)	Cadaverine (5mM)
10min.	Without Mg^{2+}	3·6	3·0	2·6	3·6	3·1
(Expt. 1)	With Mg^{2+} (5mM)	6·1	12·3	29·3	13·4	13·0
120min.	Without Mg^{2+}	−0·8	0	0	0	0
(Expt. 2)	With Mg^{2+} (5mM)	−1·5	30·1	26·6	24·0	23·3

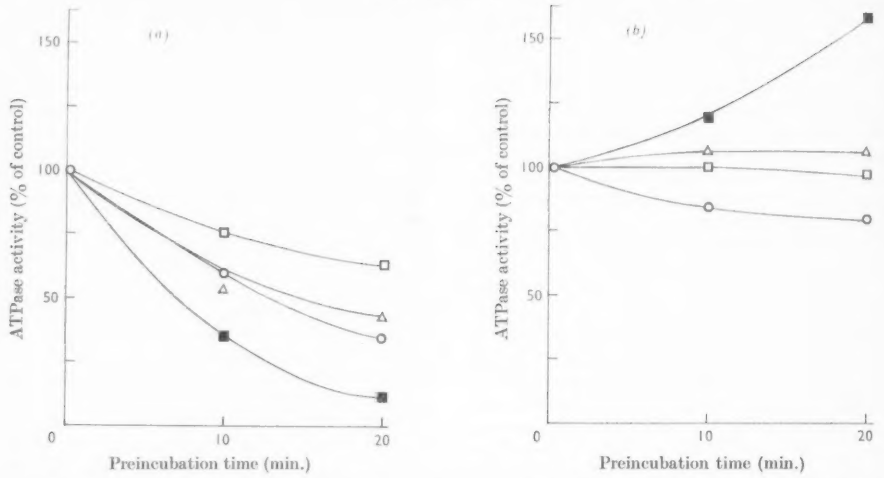

Fig. 2. (*a*) Significance of the preincubation time for the inhibitory effect of cystamine on dinitrophenol-stimulated ATPase. Intact mitochondria were preincubated in the reaction tubes at 30° for various times. ATP (5 μmoles) was then added, and the incubation continued for 10 min. Additions during the preincubation and assay period: ○, dinitrophenol (0·25 mM); ■, dinitrophenol (0·25 mM) + cystamine (5 mM); △, dinitrophenol (0·25 mM) + cysteamine (10 mM); □, dinitrophenol (0·25 mM) + cadaverine (5 mM). The values are given as percentages of those of controls with mitochondria added at the start of the assay period. The activity of the controls with dinitrophenol was 15 μmoles of P_i liberated/mg. of protein/hr. (*b*) Significance of the preincubation time for the stimulation by cystamine of the ATPase assayed in the presence of dinitrophenol and Mg^{2+}. The experimental conditions were the same as those described in the legend to Fig. 2(*a*), except that Mg^{2+} (5 mM) was present in all tubes during preincubation and assay period. The symbols are those used in Fig. 2(*a*).

cadaverine. The inhibitory effect of cystamine on dinitrophenol-stimulated ATPase could be completely reversed by the addition of Mg^{2+} (Table 1 and Fig. 2*b*). These observations suggested that the disulphide caused an accelerated loss of Mg^{2+} from the mitochondria.

In a series of experiments where the duration of the preincubation period was varied, the stimulation of ATPase by cystamine in the presence of dinitrophenol and Mg^{2+} was found to be increased when the preincubation period was prolonged to 20 min. (Fig. 2*b*). Cysteamine or cadaverine did not cause any significant stimulation in the presence of dinitrophenol and Mg^{2+}, but tended to counteract the decrease of enzyme activity occurring when only the latter agents were present. This decrease is in accordance with observations by Potter, Siekevitz & Simonson (1953).

Fig. 3 shows that freshly prepared rat-liver mitochondria, when incubated at 30° in a tris–sucrose medium, lost Mg^{2+}. The loss was usually less than 30% after 15 min., however. Cystamine caused a pronounced increase of the Mg^{2+} loss, leaving no detectable Mg^{2+} in the mitochondria after 15–20 min. (Fig. 3). A similar, but less pronounced,

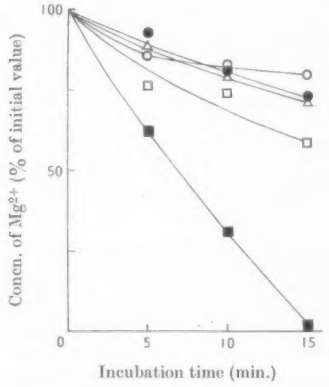

Fig. 3. Release of Mg^{2+} from mitochondria incubated in the presence of cystamine, cysteamine, cadaverine or dinitrophenol. Experimental details are given in the Methods section. The values are given as percentages of the amount of Mg^{2+} initially present (33–40 mμmoles of Mg^{2+}/mg. of mitochondrial protein). The additions were: ●, none; □, dinitrophenol (0·25 mM); ■, cystamine (5 mM); △, cysteamine (10 mM); ○, cadaverine (5 mM).

effect was elicited by dinitrophenol. Cysteamine did not influence the release of Mg^{2+}. Cadaverine retarded the loss slightly. In uncited experiments it was shown that the combination of cystamine and dinitrophenol did not cause a loss of Mg^{2+} more rapid than that obtained with cystamine alone.

Studies on disrupted mitochondria were undertaken to elucidate whether cystamine has any direct stimulating effect on the ATPase activity. Mitochondria disrupted by a short exposure to ultrasonic vibrations evidently had lost their Mg^{2+}, as they did not exhibit any significant ATPase activity without the addition of Mg^{2+} (Table 3). The addition of dinitrophenol, cystamine or cysteamine to such preparations did not elicit any significant ATPase activity. However, the activating effect of dinitrophenol was in some experiments still observable in ultrasonically treated mitochondria in the presence of Mg^{2+}. These results are comparable with those obtained by Lardy & Wellman (1953) with fresh mitochondria and acetone-dried powder extracts, or with those obtained by Cooper (1958) with 'digitonin particles', and are in accordance with the concept that dinitrophenol acts by stimulating Mg^{2+}-activated ATPase. No further stimulation by cystamine or cysteamine in addition to that obtained with dinitrophenol and Mg^{2+} was observed in disrupted mitochondria.

Table 4 shows that cystamine was a moderate inhibitor of the $[^{32}P]P_i$–ATP exchange reaction, giving a 40% inhibition at 5mM. Cysteamine, on the other hand, did not give any significant inhibition of the exchange reaction. The inhibitory effect of cystamine was very moderate, in contrast with the effect of dinitrophenol, which is indicated for comparison. Dinitrophenol gave a nearly complete inhibition at concentrations as low as 0·05mM.

It has been reported that cysteamine causes uncoupling of oxidative phosphorylation (van Bekkum & de Groot, 1956; Park *et al.* 1956). Park

Table 3. *Effects of cystamine, cysteamine,* Mg^{2+} *and dinitrophenol on the ATPase activity of disrupted mitochondria*

The mitochondria were disrupted by ultrasonic vibrations. The protein content of each tube was approx. 0·5mg./2ml. The incubations were performed at 30° for 10min. after temperature equilibration for 5min. The concentrations of the additions were: cystamine, 5mM (Expt. 1); cysteamine, 10mM (Expt. 2); $MgCl_2$, 5mM; dinitrophenol, 0·13mM; ATP, 5mM.

Expt. 1 (with cystamine)		Expt. 2 (with cysteamine)	
Addition	ATPase activity (μmoles/hr./mg. of protein)	Addition	ATPase activity (μmoles/hr./mg. of protein)
None	0·2	None	0·0
Cystamine	0·6	Cysteamine	1·4
Dinitrophenol	0·7	Dinitrophenol	0·9
Dinitrophenol + cystamine	1·4	Dinitrophenol + cysteamine	1·0
Mg^{2+}	15·8	Mg^{2+}	20·3
Mg^{2+} + cystamine	15·6	Mg^{2+} + cysteamine	16·2
Mg^{2+} + dinitrophenol	18·5	Mg^{2+} + dinitrophenol	19·5
Mg^{2+} + dinitrophenol + cystamine	18·6	Mg^{2+} + dinitrophenol + cysteamine	21·0

Table 4. *Effects of cystamine and cysteamine on mitochondrial* $[^{32}P]P_i$*–ATP exchange activity*

Experimental details are given in the Methods section. The experiment was started with the addition of mitochondria and was run for 10min. at 30°. The results are given as loss in activity as a percentage of that in uninhibited controls. The exchange activity of the controls was in the range 0·05–0·1 μmole of P_i incorporated into ATP/min./mg. of protein.

Addition	Inhibition (%)						
Concn. of addition (mM)	0·05	0·5	1·0	2·5	5·0	10·0	20·0
Cystamine	—	12	8	24	41	66	—
Cysteamine	—	0	0	0	0	5	5
Dinitrophenol	93	98	99	—	—	—	—

Table 5. *Effects of cystamine and cysteamine on oxidative phosphorylation*

Rat-liver mitochondria (approx. 9 mg. of protein; 7 mg. in Expt. 3) were used with succinate (20 μmoles) as substrate in Warburg experiments. In Expts. 2 and 4, mitochondria were preincubated in the main chamber for 7 min. Cystamine or dinitrophenol was then added from the side arm together with substrate, hexokinase and glucose. In Expts. 1 and 3, the mitochondria were preincubated for 7 min. with cystamine or cysteamine as indicated. The reaction was then run for 10 min. (Expts. 1, 2 and 4) or 15 min. (Expt. 3).

Addition	Concn. of addition (mM).....	0	0·05	0·5	1·0	2·5	5·0	10·0
Cystamine (Expt. 1)	P_i esterified (μmoles)	21·7	—	18·0	12·4	2·7	0	0
	O_2 uptake (μg.atoms)	10·3	—	9·9	7·6	7·7	7·4	7·1
	P/O ratio	2·1	—	1·8	1·6	0·3	0	0
Cystamine (Expt. 2)	P_i esterified (μmoles)	18·1	—	17·0	14·0	16·7	16·5	14·5
	O_2 uptake (μg.atoms)	9·7	—	9·6	10·0	9·0	10·3	9·6
	P/O ratio	1·9	—	1·8	1·4	1·8	1·6	1·5
Cysteamine (Expt. 3)	P_i esterified (μmoles)	21·4	—	—	—	—	20·9	17·5
	O_2 uptake (μg.atoms)	12·0	—	—	—	—	11·6	9·8
	P/O ratio	1·8	—	—	—	—	1·8	1·8
Dinitrophenol (Expt. 4)	P_i esterified (μmoles)	14·9	2·5	—	—	—	—	—
	O_2 uptake (μg.atoms)	8·4	4·3	—	—	—	—	—
	P/O ratio	1·8	0·6	—	—	—	—	—

et al. (1956) report that cysteamine produces a detectable uncoupling at a concentration of 1 mM, and that cystamine uncouples at concentrations 50-fold lower. They suggest that the uncoupling observed with cysteamine might be due to cystamine formed by the autoxidation of the thiol. Oxidation of cysteamine occurs easily at pH 7·4 and 30°. In my experiments, after 15 min. of incubation of a freshly prepared cysteamine solution (5 or 10 mM) 5–10% of the thiol was already oxidized. In the experiments given in Table 5, the tendency of cysteamine to autoxidize was counteracted by the addition of thiolated Sephadex (Jellum, 1964). No uncoupling by cysteamine could then be observed, in accordance with the observation that the concentration of thiol was unchanged at the end of the experimental period.

Mitochondria reduce several small-molecular disulphides in the presence of added citric acid-cycle intermediates (Eldjarn & Bremer, 1963). If the true effect of cystamine on oxidative phosphorylation is to appear, conditions must therefore be chosen to ensure that the disulphide is not reduced by the mitochondria. This is the case when the mitochondria are preincubated with cystamine for 5–10 min. Their disulphide-reducing capacity is then lost (Skrede *et al.* 1965). In Expt. 1 in Table 5, the mitochondria were preincubated with cystamine for 7 min. in the absence of substrate, and it is shown that this disulphide caused a complete uncoupling at 5 mM. Under the conditions of Expt. 1 in Table 5, cystamine also caused a moderate inhibition of the oxygen uptake. Possibly, cystamine, by analogy with dinitrophenol (see also Expt. 4 in Table 5), because of its uncoupling effects, caused an accumulation of oxaloacetate (Slater & Hülsmann, 1961), which is a powerful inhibitor of succinate oxidation.

Under conditions where the mitochondria were able to reduce a significant part of the disulphide to cysteamine, which does not cause uncoupling, only a slight uncoupling effect could be seen (Expt. 2 in Table 5). In the latter experiment, cystamine and succinate were added to the mitochondria simultaneously at the start of the experimental period after the usual temperature equilibration for 7 min.

DISCUSSION

It has been discussed whether the ATPase unmasked by 2,4-dinitrophenol (Lardy & Wellman, 1953) and the ATPase revealed by Mg^{2+} in aged mitochondria (Kielley & Kielley, 1951) are different entities (Racker, 1961; Lehninger & Wadkins, 1962). Mg^{2+} seem to be necessary also for the stimulation of ATPase by dinitrophenol (Lehninger & Wadkins, 1962). Therefore dinitrophenol-stimulated ATPase and Mg^{2+}-activated ATPase seem to have at least some step in common (Siekevitz *et al.* 1958), or may represent the same enzyme system activated in different ways (Cooper, 1958; Racker, 1961). The role of Mg^{2+} in the activation process is not clear, but probably a chelate between Mg^{2+} and ATP is the 'active substrate' for the enzyme (Kielley, 1951; Cooper & Lehninger, 1957; Ulrich, 1964).

In the present study, the following effects of

cystamine were shown: (1) an unmasking of the ATPase activity of fresh mitochondria; (2) a release of Mg^{2+} from the mitochondria; (3) a decrease in the activation of the ATPase activity of fresh mitochondria by dinitrophenol in the absence of Mg^{2+}; (4) a stimulation of the activation of the ATPase of fresh mitochondria by dinitrophenol in the presence of Mg^{2+}; (5) uncoupling of oxidative phosphorylation; (6) an inhibition of the $[^{32}P]P_i$–ATP exchange reaction.

In these investigations, the effects of cystamine were compared with those of the chemically closely related diamine cadaverine and the corresponding thiol cysteamine. Since the latter two compounds did not show the listed effects, cystamine probably acts by its disulphide group.

The above effects of cystamine may appear to be rather complex. However, it might prove profitable to discuss all observations from the angle that the effects may be due to primary structural changes in the mitochondria caused by the disulphide. This explanation gains some support from a previous observation that cystamine stimulates choline oxidation by fresh mitochondria, and inhibits nicotinamide nucleotide-linked oxidations, probably by causing a loss of nicotinamide nucleotides from the mitochondria (Skrede *et al.* 1965). Other authors have shown that a number of surface-active agents activate the ATPase of fresh mitochondria by causing damage to the mitochondrial structure; such compounds are digitonin (Cooper & Lehninger, 1956), bilirubin and deoxycholate (Pressman & Lardy, 1956; Siekevitz *et al.* 1958). The unmasking of the ATPase of fresh mitochondria by cystamine may also well be explained by such a mechanism. One of the most striking observations in the present study is the accelerated Mg^{2+} loss from the mitochondria during incubation with cystamine. Under normal conditions, about half of the Mg^{2+} of mitochondria is tightly bound to the mitochondrial structure (Lehninger, 1964), but the nature of the binding mechanism is not known. That cystamine causes a leakage of Mg^{2+} from the mitochondria suggests that this disulphide reacts with thiol groups involved in the maintenance of mitochondrial morphology, first studied by Tapley (1956). Such thiol groups are apparently required for the retention of Mg^{2+}, even that part which is tightly bound. In the present study, cadaverine was shown to retard the loss of Mg^{2+} slightly, and accordingly to delay the inactivation of dinitrophenol-stimulated ATPase which appears on prolonged preincubation with dinitrophenol. These effects of cadaverine are probably analogous to the effects of spermine on mitochondria. The latter polyamine, by an unknown mechanism, offers protection against swelling and loss of material absorbing at 260mμ from the mitochondria (Tabor, Tabor & Rosenthal, 1961).

The importance of permeability effects in the study of mitochondrial ATPase has been pointed out (Perry & Chappell, 1957), as fresh mitochondria may have a limited permeability to ATP (Siekevitz & Potter, 1955*b*). This view is supported by observations suggesting that, in fresh mitochondria, the combined effect of Mg^{2+} and an agent which causes an increased permeability of the membrane(s) seems to be necessary to give maximal ATPase activity. Thus Potter *et al.* (1953) observed maximal stimulation of the ATPase of fresh mitochondria when Ca^{2+} was added together with Mg^{2+}. Ca^{2+} is (like thyroxine) believed to give primary structural changes of the mitochondria (Tapley & Cooper, 1956). In the present experiments, maximal stimulation of the ATPase activity of fresh mitochondria was obtained with cystamine and dinitrophenol in the presence of Mg^{2+}, whereas dinitrophenol and Mg^{2+}, in accordance with the observations by Potter *et al.* (1953), caused a submaximal stimulation (Expt. 1 in Table 1 and Fig. 2*b*). Also, these findings may well be explained by membrane effects of cystamine, allowing a facilitated diffusion of ATP and Mg^{2+} to the ATP-hydrolysing sites in the mitochondria.

An attempt was made to eliminate some of the effects of cystamine on mitochondrial permeability by studying the interference of this disulphide with mitochondrial ATPase in ultrasonically disrupted mitochondria. Such particles had apparently lost their Mg^{2+}. The results also indicated that cystamine is not a direct activator of mitochondrial ATPase, and that the disulphide does not interfere with the activation of the ATPase of such mitochondrial particles by Mg^{2+}. From these results, some conclusions on the effects of cystamine apart from the effects on mitochondrial permeability may be suggested. In a study with thiol-binding agents, Cooper (1960) showed that ATPase obtained from digitonin extracts of mitochondria has one 'inhibitory' set of thiol groups, as well as another set essential for the activity of the enzyme. The present observation that the ATPase activity of ultrasonically disrupted mitochondria is not changed by cystamine also suggests that neither of these two sets of thiol groups of mitochondrial ATPase possesses significant reactivity towards cystamine. This finding was not very surprising, however, since this disulphide is of a low oxidation potential, whereas the inhibitory set of thiol groups of the ATPase of mitochondrial particles obtained by ultrasonic oscillations are of a relatively low reactivity as judged from experiments with *p*-chloromercuribenzoate (Kielley, 1963).

When the above effects of cystamine are compared with those of dinitrophenol, it seems likely

that these agents act by distinctly different mechanisms. Cystamine has only a moderate effect on oxidative phosphorylation, ATPase activity of fresh mitochondria and $[^{32}P]P_i$–ATP exchange, when compared with dinitrophenol. On the other hand, the release of Mg^{2+} from the mitochondria is accelerated more by cystamine than by dinitrophenol, and cystamine is also required for the maximal stimulation of the ATPase of fresh mitochondria.

As a conclusion, it appears likely that, whereas dinitrophenol acts on the chain of oxidative phosphorylation by reacting with a 'high-energy' intermediate (Hemker, 1964), cystamine diminishes mitochondrial permeability barriers. The uncoupling effect of cystamine may be more 'indirect' and mediated by an effect on the mitochondrial structure, resembling the effects of thyroxine or Ca^{2+} (Tapley & Cooper, 1956). However, the effects of cystamine on mitochondrial permeability obviously cannot be directly related to its swelling effects. Thus the thiol cysteamine is nearly as effective as cystamine as a swelling agent (Neubert & Lehninger, 1962). This observation has been confirmed in this Laboratory (unpublished results), with special precautions taken to prevent the autoxidation of cysteamine to cystamine.

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Some Factors affecting the Response of Muscle to Insulin

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1. The effect of various changes in the composition of the supporting medium on the capacity of isolated rat diaphragm to incorporate amino acids into its protein has been studied. 2. Replacement of most of the normal ionic constituents by sucrose is inhibitory towards protein synthesis, as is also substitution of choline or K^+ for Na^+. 3. The capacity of the tissue to respond to a stimulatory effect of insulin is impaired in the sucrose media and under certain conditions in the absence of Na^+, particularly when Na^+ is replaced by K^+ and the ^{14}C-labelled amino acid is presented at a relatively high concentration. 4. Cutting of the tissue before incubation also decreases incorporating capacity and markedly decreases responsiveness to insulin. 5. In abnormal media the cellular content of ATP falls sharply. 6. The ATP content of the tissue also declines in the presence of 2-deoxyglucose. This change is prevented by the addition of glucose but not of pyruvate and succinate. 7. Although affecting the rate of amino acid incorporation the ATP content is not thought generally to limit sensitivity to insulin.

Although insulin *in vitro* will very consistently stimulate a number of metabolic activities of isolated rat diaphragm muscle, particularly uptake of sugar, incorporation of amino acids into protein and accumulation of amino acids (for review see Manchester, 1965), there have been a number of papers recently describing conditions of incubation under which normal effects of insulin are no longer seen.

Bhattacharya (1959*a*,*b*, 1961) found that replacement of much of the normal ionic composition of the incubation medium for diaphragm by sucrose interfered with the stimulation by insulin of glucose uptake. However, Mg^{2+} appeared to possess a unique role of preserving sensitivity to insulin. Manchester (1961*a*) showed that replacement of Na^+ by K^+ interfered with incorporation of amino acids into protein of diaphragm muscle and its stimulation by insulin. However, Kostyo (1964), in a study of the action of pituitary growth hormone on diaphragm, found that when Na^+ was replaced by choline, although the amount of amino acid incorporation declined, stimulation of incorporation by insulin was still observable. In a slightly different context work by Fong, Silver, Popenoe & Debons (1962) and others (Cadenas, Kaji, Park & Rasmussen, 1961; Carlin & Hechter, 1962; Mirsky & Perisutti, 1962; Edelman, Rosenthal & Schwartz, 1963) has suggested that a primary point of interaction of insulin with responsive tissues is through thiol groups and the response of a tissue to insulin can be modified by agents affecting such groups. DeSchepper, Toyoda & Bessman (1965) have reported the inhibition of amino acid incorporation into protein of diaphragm, and its stimulation by insulin, by the addition of 2-deoxyglucose to the incubation medium.

A determination of the precise factors in an intact tissue necessary for it to be capable of responding to insulin would assist in understanding how precisely insulin regulates in muscle the transport of sugars and the accumulation and incorporation of amino acids into protein. In particular, it would be valuable to know the degree of derangement of structure and organization of a tissue that can be tolerated before its ability to respond to insulin is lost. The present paper examines some of the parameters regulating the capacity of muscle to respond to the stimulating influence of insulin on the incorporation of amino acids into protein.

MATERIALS AND METHODS

Diaphragm muscle was taken from non-starved female rats, about 100g. body wt., and incubated with shaking for 2hr. at 37° as described by Manchester (1961*b*). The intact diaphragm preparation (retaining the rib-cage) was that of Kipnis & Cori (1957). The medium for incubation was Krebs–Ringer bicarbonate buffer (Krebs & Henseleit, 1932) or various modifications. The composition of the buffers is stated in Table 1. Replacement of Na^+ by K^+ means that the Krebs–Ringer bicarbonate buffer contained KCl in place of NaCl and $KHCO_3$ instead of $NaHCO_3$. Choline buffer had choline chloride in place of NaCl and $KHCO_3$ instead of $NaHCO_3$. In potassium methyl sulphate buffer,

potassium methyl sulphate replaced NaCl, calcium acetate replaced $CaCl_2$ and $KHCO_3$ replaced $NaHCO_3$. Unless otherwise stated no glucose was added to the media. All solutions were gassed with $CO_2 + O_2$ (5:95) and this was the gas phase throughout the incubation. Radioactive amino acids, from The Radiochemical Centre, Amersham, Bucks., were all L-isomers and uniformly labelled with ^{14}C except for [1-^{14}C]glycine. They were added to the medium unless otherwise stated at about 1 μc/6·5 ml. (Tables 4–7) or at about 1 μl./12·5 ml. (Tables 2 and 9), either undiluted or diluted with unlabelled amino acid to a concentration of 1 mM.

At the end of the incubation the tissue was placed in 10% (w/v) trichloroacetic acid and protein samples were prepared for counting as described by Manchester (1961*b*) except for a minor modification in the plating technique. After washing the protein with acetone the material was either suspended in ether and poured on to tared aluminium planchets (experiments in Tables 2 and 9) or dissolved in formic acid and half the available material pipetted on to a concentric ringed stainless-steel planchet (experiments in Tables 4–6 and 8). The results in the different Tables are therefore not strictly comparable. The content of radioactivity was assessed in a Nuclear–Chicago thin end-window gas-flow counter having an efficiency of about 25%. Since the sample weights in any given experiment were fairly constant no corrections for self-absorption were made in calculating specific activities.

Accumulation of amino acids was measured as described by Manchester & Young (1960).

ATP was measured by an enzymic method based on phosphoglycerate kinase (EC 2.7.2.3). The tissue was ground in ice-cold 5% (v/v) $HClO_4$ and the $HClO_4$ neutralized and precipitated by the addition of a mixture of KOH and potassium acetate. A sample of supernatant was added to an assay medium consisting of tris (0·1 M), EDTA (10 mM), $MgSO_4$ (9 mM), hydrazine sulphate (3·5 mM), phosphoglycerate (8·5 mM) and NADH (0·2 mM), final pH 7·5. The decrease in the extinction at 340 mμ was followed after the addition of 100 μg. of glyceraldehyde phosphate dehydrogenase (EC 1.2.1.12) (to remove diphosphoglycerate), followed by 40 μg. of phosphoglycerate kinase. Both enzymes were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany.

RESULTS

Role of Mg^{2+}. The first experiments were carried out to see whether Mg^{2+} affects the stimulation by insulin of incorporation of amino acids into protein of diaphragm muscle as it influences sugar transport (Bhattacharya, 1959*a*,*b*, 1961). Incorporation of radioactivity in the absence of insulin was as great in Krebs–Ringer phosphate buffer (II) or Stadie–Zapp medium (III) (Stadie & Zapp, 1947) as in Krebs–Ringer bicarbonate buffer (I) (Table 2). Replacement of Krebs–Ringer bicarbonate buffer by a sodium chloride–bicarbonate buffer (IV) brought about some diminution in glycine incorporation. The addition of insulin (0·1 unit/ml.) to each buffer brought about a marked stimulation of incorporation. Replacement of Mg^{2+} and Ca^{2+} by Na^+ (buffer V) decreased incorporation slightly, though replacement of only Mg^{2+} by Na^+ (buffer VI) had no similar effect. Neither change resulted in diminution of response to insulin. Replacement of all Na^+ by K^+ (buffer VII) markedly decreased incorporation and abolished the effect of insulin, but partial reintroduction of Na^+ (buffer VIII) led to restoration of the rate of incorporation and to recovery of sensitivity to insulin. Incorporation and its enhancement by insulin was markedly curtailed when diaphragm was incubated in a sucrose–bicarbonate medium (IX) similar to that used by Bhattacharya (1961) or in a similar medium compounded of sorbitol buffered with sodium hydrogen carbonate or potassium hydrogen carbonate (not shown in Table 2). As Bhattacharya (1961) showed for the uptake of glucose, sensitivity to stimulation by insulin of incorporation of amino acids into protein is enhanced by Mg^{2+} when the tissue is incubated in a buffer containing substantial amounts of sucrose. However, in buffers of more physiological ionic composition a normal response

Table 1. *Composition of buffers used for the incubation of isolated rat diaphragm*

Further details are given in the text. These buffers were used in the experiments described in subsequent Tables.

	Composition (μmoles/ml.)								
Buffer	Na^+	K^+	Mg^{2+}	Ca^{2+}	Cl^-	$H_2PO_4^-$–HPO_4^{2-}	HCO_3^-	SO_4^{2-}	Sucrose
I	143	6	1·2	2·5	128	1·2	25	1·2	—
II	151	5	1·2	2·5	133	16	—	1·2	—
III	140	—	5	—	80	40	—	—	—
IV	154	—	—	—	128	—	26	—	—
V	147	6	—	—	128	1·2	25	1·2	—
VI	144	6	—	2·5	128	1·2	25	1·2	—
VII	—	150	1·2	2·5	128	1·2	25	1·2	—
VIII	25	124	1·2	2·5	128	1·2	25	1·2	—
IX	25	—	—	—	—	—	25	—	250

Table 2. *Effect of alteration of ionic composition of the incubation medium on the incorporation of [^{14}C]glycine into the protein of isolated rat diaphragm and the stimulation of this process by insulin*

Incubation was for 2 hr. in the media indicated whose composition is described in Table 1. [1-^{14}C]Glycine was present at a concentration of 1 mM and about 80 μc/m-mole. Each result is the mean ± S.E.M. of the number of observations indicated.

Incubation buffer	No. of observations	Radioactivity in diaphragm protein (counts/min./mg.) No insulin added	Insulin added (0·1 unit/ml.)	Percentage increase	P
1 (a) Krebs–Ringer bicarbonate buffer (I)	6	18·7 ± 1·05	31·4 ± 2·48	69	< 0·001
(b) Krebs–Ringer phosphate buffer, pH 7·2 (II)	6	18·9 ± 1·79	30·4 ± 2·53	61	< 0·001
(c) Stadie–Zapp buffer, pH 7·2 (III)	6	18·2 ± 1·19	31·6 ± 2·31	74	< 0·001
2 (a) Krebs–Ringer bicarbonate buffer (I)	12	22·8 ± 1·36	36·9 ± 2·48	62	< 0·001
(b) NaCl–$NaHCO_3$ buffer (IV)	12	19·2 ± 0·38 (a)–(b) P < 0·02	29·6 ± 0·91 (a)–(b) P < 0·001	54	< 0·001
3 (a) Krebs–Ringer bicarbonate buffer (I)	6	16·0 ± 0·74	—		
(b) Buffer (I) with all Mg^{2+} and Ca^{2+} replaced by Na^+ (V)	6	13·2 ± 1·24	22·3 ± 0·72	69	< 0·001
4 (a) Krebs–Ringer bicarbonate buffer (I)	4	14·6 ± 0·76	—		
(b) Buffer (I) with all Mg^{2+} replaced by Na^+ (VI)	4	15·5 ± 0·43	20·7 ± 2·10	34	= 0·05
5 (a) Krebs–Ringer bicarbonate buffer (I)	6	19·7 ± 1·69	—		
(b) Buffer (I) with all Na^+ replaced by K^+ (VII)	6	6·8 ± 0·52	6·5 ± 0·42	−4	
(c) Buffer (I) with 118 m-equiv. of Na^+ replaced by K^+ (VIII)	6	11·9 ± 0·49 (a)–(b) P < 0·001 (a)–(c) P < 0·01 (c)–(b) P < 0·001	15·7 ± 1·15 (c)–(b) P < 0·001	32	= 0·02
6 (a) Krebs–Ringer bicarbonate buffer (I)	12	20·2 ± 1·24	31·8 ± 2·12	57	< 0·001
(b) Sucrose–$NaHCO_3$ buffer (IX) + NaCl (1 mM)	12	11·7 ± 0·51	12·8 ± 0·42	9	
(c) Sucrose–$NaHCO_3$ buffer (IX) + $MgCl_2$ (1 mM)	12	14·7 ± 0·99 (a)–(b) P < 0·001 (a)–(c) P < 0·01 (c)–(b) P < 0·02	20·0 ± 1·18 (a)–(b) P < 0·001 (a)–(c) P < 0·001 (c)–(b) P < 0·001	36	< 0·01
7 (a) Sucrose–$NaHCO_3$ buffer (IX) + NaCl (1 mM)	12	10·9 ± 0·59	—		
(b) Sucrose–$NaHCO_3$ buffer (IX) + KCl (1 mM)	12	10·3 ± 0·57	—		
(c) Sucrose–$NaHCO_3$ buffer (IX) + $MgCl_2$ (1 mM)	12	15·2 ± 0·85	—		
(d) Sucrose–$NaHCO_3$ buffer (IX) + $CaCl_2$ (1 mM)	12	12·8 ± 0·91	—		
(e) Sucrose–$NaHCO_3$ buffer (IX) + $MnCl_2$ (1 mM)	12	8·8 ± 0·41	—		
(f) Sucrose–$NaHCO_3$ buffer (IX) + $ZnCl_2$ (1 mM)	12	9·0 ± 0·55 (c)–(a) P < 0·001	—		

to insulin is observed in the absence of added Mg^{2+} and there seems no reason to suppose that under these conditions this is especially involved in the response of muscle to the hormone. The results of Table 2, for the incorporation of amino acid into protein, reveal what is not obvious from the study of glucose uptake, namely the inadequacy of a sucrose or sorbitol–bicarbonate medium as a supporting medium for diaphragm muscle as judged by the lower rate of amino acid incorporation. Moreover, as shown in Table 3, incubation in iso-osmotic sucrose leads to shrinkage of the diaphragm. The

addition of Mg^{2+} appeared to restrain the onset of rigor during incubation in sucrose–bicarbonate medium and it seems probable that the effect of the ion on the amount of incorporation and sensitivity to insulin is a consequence of this maintenance of the condition of the tissue and its influence on the ATP content (discussed below and Table 10). The effect of Mg^{2+} on incorporation was not reproduced by other bivalent ions (Table 2).

Role of Na^+. The effect of replacement of Na^+ by K^+ is more drastic than replacement by choline because increasing the concentration of K^+ both depolarizes the cell membrane and brings about swelling of the tissue (Table 3), effects that are not seen with choline. The complexity of the system is indicated in Table 4; the loss of response to insulin in K^+ buffer occurs only when a relatively high concentration of ^{14}C-labelled amino acid is added but not when a trace concentration is used. A stimulation by insulin is always seen when the amino acid is added to the medium at a concentration of about 10–20 μM, but at 1 mM concentration of amino acid K^+ brings about a repression of the response to the hormone whereas choline does not. Incorporation of [^{14}C]glycine is decreased to a similar degree in K^+ or choline buffer (Table 5), whereas incorporation of [^{14}C]leucine is less severely decreased in choline than in K^+ buffer. In the latter medium the degree of inhibition for leucine appears to be comparable with that for glycine.

Table 3. *Effect of composition of the supporting medium on the weight of rat diaphragm muscle during incubation*

The composition of Krebs–Ringer bicarbonate buffer (I) and the various modifications is described in the Materials and Methods section, and that of the sucrose–$NaHCO_3$ buffer (IX) in Table 1. Incubation was for 2 hr. Each result is the mean ± S.E.M. of the number of observations indicated.

Incubation buffer	No. of observations	Final wet wt. (% of initial wt.)
Krebs–Ringer bicarbonate buffer (I)	6	97·3 ± 0·41
Buffer (I) with Na^+ replaced by choline	6	99·5 ± 1·46
Buffer (I) with Na^+ replaced by K^+	12	120 ± 1·66
Buffer (I) with Na^+ replaced by K^+ and Cl^- by $MeSO_4^-$	12	108 ± 0·82
Sucrose–$NaHCO_3$ buffer (IX)	6	83·3 ± 0·95
Sucrose–$NaHCO_3$ buffer (IX) + $MgCl_2$ (1 mM)	6	86·5 ± 2·26

Table 4. *Effect of insulin on the incorporation of* ^{14}C*-labelled amino acids into the protein of diaphragm incubated in buffers containing no* Na^+

The incubation buffer had the same composition as Krebs–Ringer bicarbonate buffer (I) except that all the Na^+ had been replaced by choline or K^+ as described in the Materials and Methods section. Incubation was for 2 hr. Each result is the mean ± S.E.M. of six observations.

Na^+ of incubation buffer replaced by	Amino acid	Radioactivity in diaphragm protein (counts/min./mg.) No insulin added	Insulin added (0·1 unit/ml.)	Percentage increase
Choline	Glycine (1·0 mM)	14·9 ± 0·47	20·9 ± 1·04‡	40
	Glycine (20 μM)	31·2 ± 1·12	36·8 ± 1·88*	18
	Proline (1·0 mM)	16·8 ± 0·56	23·0 ± 1·78†	37
	Proline (13 μM)	99 ± 4·2	126 ± 7·2†	27
	Phenylalanine (1·0 mM)	18·1 ± 0·94	22·4 ± 0·84†	24
	Phenylalanine (21 μM)	41·4 ± 2·34	53·4 ± 2·80†	23
K^+	Glycine (1·0 mM)	14·7 ± 1·31	15·7 ± 2·07	7
	Glycine (20 μM)	23·8 ± 1·13	30·5 ± 1·50†	28
	Proline (1·0 mM)	18·6 ± 1·12	19·4 ± 1·48	4
	Phenylalanine (1·0 mM)	20·2 ± 0·60	20·8 ± 1·20	3
	Phenylalanine (21 μM)	57·0 ± 1·88	64·4 ± 2·90*	31
	Leucine (1·0 mM)	70·6 ± 3·48	69·6 ± 4·60	−2
	Leucine (19 μM)	332 ± 27	436 ± 31*	31

* $P < 0·05$; † $P < 0·01$; ‡ $P < 0·001$.

Table 5. *Effect of various changes in the composition of the incubation medium on the basal rate of incorporation of amino acids into the protein of isolated rat diaphragm*

The composition of Krebs–Ringer bicarbonate buffer (I) and the various modifications is described in the Materials and Methods section. Incubation was for 2 hr. Each result is the mean ± S.E.M. of six observations.

Amino acid	Incubation buffer	Radioactivity in diaphragm protein (counts/min./mg.)	
Leucine (19 μM)	Krebs–Ringer bicarbonate buffer (I)	827 ± 77	$P < 0.01$
	Buffer (I) with Na^+ replaced by choline	530 ± 22	$P < 0.001$
	Buffer (I) with Na^+ replaced by K^+	312 ± 22	
Leucine (1·0 mM)	Buffer (I) with Na^+ replaced by choline	113 ± 4·3	$P < 0.001$
	Buffer (I) with Na^+ replaced by K^+	76 ± 10·0	
Glycine (20 μM)	Buffer (I) with Na^+ replaced by choline	29·0 ± 1·42	
	Buffer (I) with Na^+ replaced by K^+	28·5 ± 1·30	
Glycine (1·0 mM)	Buffer (I) with Na^+ replaced by choline	16·3 ± 1·55	
	Buffer (I) with Na^+ replaced by K^+	15·7 ± 0·93	

Table 6. *Effect of potassium methyl sulphate as a supporting salt on the incorporation of amino acids into protein by isolated rat diaphragm*

Incubation buffers had the same composition as Krebs–Ringer bicarbonate buffer (I) except where indicated that Na^+ had been replaced by K^+, and/or Cl^- by $MeSO_4^-$, as described in the Materials and Methods section. The final concentration of amino acid added was 1 mM; incubation was for 2 hr. Each result is the mean ± S.E.M. of six observations.

Amino acid	Incubation buffer	Radioactivity in diaphragm protein (counts/min./mg.)		Percentage increase
		No insulin added	Insulin added (0·1 unit/ml.)	
Glycine	Buffer (I) with Na^+ replaced by K^+	16·7 ± 2·85	16·5 ± 1·16	−1
	Buffer (I) with Na^+ replaced by K^+ and Cl^- by $MeSO_4^-$	31·4 ± 2·83	36·3 ± 1·24	12
Leucine	Krebs–Ringer bicarbonate buffer (I)	261 ± 11·1	300 ± 4·4	15
	Buffer (I) with Na^+ replaced by K^+ and Cl^- by $MeSO_4^-$	158 ± 12·2	162 ± 8·4	2·5

Table 7. *Effect of replacement of Na^+ by K^+ or choline on the accumulation of amino acids by isolated rat diaphragm*

Incubation buffers had the same composition as Krebs–Ringer bicarbonate buffer (I) except where indicated that Na^+ had been replaced by choline or K^+ as described in the Materials and Methods section. Incubation was for 2 hr. Each result is the mean ± S.E.M. of six observations.

Amino acid	Incubation buffer	Radioactivity of tissue water (counts/min./ml.) / Radioactivity of medium (counts/min./ml.)	
		No insulin added	Insulin added (0·1 unit/ml.)
Glycine (1·0 mM)	Krebs–Ringer bicarbonate buffer (I)	3·60 ± 0·14	4·40 ± 0·37
	Buffer (I) with Na^+ replaced by K^+	1·03 ± 0·029	1·08 ± 0·024
Leucine (1·0 mM)	Krebs–Ringer bicarbonate buffer (I)	3·66 ± 0·20	3·65 ± 0·16
	Buffer (I) with Na^+ replaced by K^+	1·08 ± 0·019	1·10 ± 0·021
Glycine (20 μM)	Buffer (I) with Na^+ replaced by choline	1·38 ± 0·023	1·42 ± 0·025

The swelling of muscle on incubating in a high-K^+ medium can be decreased somewhat, though not completely, by replacing Cl^- by the methyl sulphate anion (Table 3). In such buffer the basal level of incorporation is higher than in the presence of potassium chloride, but is still depressed by comparison with normal conditions (Table 6). In the presence of potassium methyl sulphate there is an equivocal response to insulin (Table 6).

If, as suggested by Parrish & Kipnis (1964), active transport of amino acids in muscle is dependent on and linked to the 'sodium pump', it is reasonable to suppose that interference with its functioning would bring about a decrease in the amount of ^{14}C-labelled amino acid available in the cell for incorporation into protein. Table 7 shows that replacement of Na^+ by K^+ or choline does indeed decrease the amount of accumulation of both glycine and leucine in the tissue water.

That retardation of accumulation can lessen incorporation (see Kostyo, 1964) can be demonstrated with the intact diaphragm preparation (where the tissue is retained in the rib-cage during incubation). Accumulation of glycine and aminoisobutyric acid occurs more slowly in this preparation than in the isolated hemidiaphragm (Kipnis & Noall, 1958; Manchester & Young, 1960) and in consequence (Table 8) incorporation of [^{14}C]glycine is less in the former preparation than in the latter. For leucine and phenylalanine, as opposed to glycine, whose rates of accumulation are more comparable in the two preparations (Manchester & Young, 1960), the rate of incorporation is less in the isolated hemidiaphragm, i.e. a damaged preparation, than in the rib-cage preparation. This trend is perhaps the more normal, for cutting diaphragm into strips and small pieces leads to a substantial diminution in the capacity to incorporate amino

Table 8. *Incorporation of ^{14}C-labelled amino acids into protein by the 'intact' and isolated hemidiaphragm muscle preparations*

Incubation was for 2 hr. in Krebs–Ringer bicarbonate buffer (I). Each amino acid was present at a concentration of 1 mM and about 0·125 μc/ml. Each result is the mean ± S.E.M. of the number of observations indicated.

Amino acid	No. of observations	Radioactivity in diaphragm protein (counts/min./mg.) Diaphragm contained in rib-cage	Isolated hemidiaphragm
Glycine	6	45 ± 2·7	61 ± 3·9†
Leucine	6	214 ± 2·4	195 ± 7·0*
Phenylalanine	12	81 ± 2·6	64 ± 3·2‡

* $P < 0·05$; † $P < 0·01$; ‡ $P < 0·001$.

Table 9. *Effect of cutting isolated rat diaphragm on its ability to incorporate ^{14}C-labelled amino acids into protein and its response to an effect of insulin on this process*

Incubation was for 2 hr. in Krebs–Ringer bicarbonate buffer (I). The ^{14}C-labelled amino acids were present at about 0·08 μc/ml. Each result is the mean ± S.E.M. of six observations.

Amino acid	Treatment	Radioactivity in diaphragm protein (counts/min./mg.) No insulin added	Insulin added (0·1 unit/ml.)	Difference
Glycine (1·0 mM)	Isolated hemidiaphragm	23·0 ± 0·73	32·5 ± 3·55*	9·5
	Each hemidiaphragm cut into three longitudinal strips	13·3 ± 0·61	16·1 ± 0·89*	2·8
	Each hemidiaphragm cut into small pieces about 1 mm.^{2}	10·7 ± 1·21	9·4 ± 1·23	−1·3
Phenylalanine (1·0 mM)	Isolated hemidiaphragm	52 ± 2·45	79 ± 3·71†	27
	Each hemidiaphragm cut into small pieces	14·2 ± 0·68	14·1 ± 0·91	−0·1
Glycine (10 μM)	Each hemidiaphragm cut into small pieces	57 ± 5·0	58 ± 5·0	1

* $P < 0·05$; † $P < 0·001$.

Table 10. *Effect of incubation in various media on the ATP content of rat diaphragm muscle*

The composition of Krebs–Ringer bicarbonate buffer (I) and the various modifications is described in the Materials and Methods section, that of the sucrose–$NaHCO_3$ buffer (IX) in Table 1. Incubation was for 2hr. Each result is the mean ± S.E.M. of six observations. (The ATP content of diaphragm muscle as prepared for incubation but before incubation is 2·5–3·0 μmoles/g. wet wt.).

Incubation buffer	Concn. of ATP (μmoles/g. wet wt.)
Krebs–Ringer bicarbonate buffer (I)	2·48 ± 0·23
Buffer (I) with Na^+ replaced by choline	1·32 ± 0·14
Buffer (I) with Na^+ replaced by K^+	0·61 ± 0·054
Buffer (I) with Na^+ replaced by K^+ and Cl^- by $MeSO_4^-$	1·45 ± 0·29
Sucrose–$NaHCO_3$ buffer (IX)	0·72 ± 0·040
Sucrose–$NaHCO_3$ buffer (IX)+ $MgCl_2$ (1 mM)	1·24 ± 0·13

Table 11. *Effect of 2-deoxyglucose and other materials on the ATP content of isolated rat diaphragm*

Incubation was for 2hr. in Krebs–Ringer bicarbonate buffer (I). Each result is the mean ± S.E.M. of six observations. The concentrations of metabolites are those used by DeSchepper *et al.* (1965).

Addition(s) to incubation buffer	Concn. of ATP (μmoles/g. wet wt.)
None	2·35 ± 0·069
Deoxyglucose (30 mM)	1·15 ± 0·056
Deoxyglucose (30 mM) + pyruvate (8·5 mM) and succinate (7·4 mM)	1·21 ± 0·061
Deoxyglucose (30 mM) + glucose (30 mM)	2·16 ± 0·203

acids, for both glycine and phenylalanine (Table 9). As the tissue becomes increasingly broken up the capacity to respond to insulin is progressively lost, and this is in conformity with the generally held view that *in vitro* actions of insulin are not readily seen in homogenates.

Effect of media on ATP content. Another factor that severely regulates the rate of incorporation of amino acids into protein is the availability of ATP. Thus agents that interfere with the respiratory chain bring about substantial inhibition of incorporation by diaphragm (Borsook, Deasy, Haagen-Smit, Keighley & Lowy, 1950; Manchester & Young, 1959). Table 10 indicates that many of the buffers used in the present study lead to a fall in the cellular content of ATP, the most severe losses being seen after incubation in the potassium chloride or sucrose–bicarbonate media. The factors responsible for these changes in ATP content are not known, but it seems likely that the diminutions are responsible for the lower rates of amino acid incorporation observed. It is noteworthy how the presence of Mg^{2+} in the sucrose–bicarbonate buffer restrains the fall in the ATP content under these conditions, an observation that correlates with the increased rate of amino acid incorporation (Table 2).

Changes in the cellular content of ATP also appear to be the explanation of the inhibition of incorporation found on the addition of 2-deoxyglucose (Wool, 1960) (Table 11), for the presence of the sugar brings about a substantial fall in the nucleotide concentration. DeSchepper *et al.* (1965) found in addition that the presence of deoxyglucose interfered with the stimulation of incorporation by insulin, but that its inhibitory effect in this respect was overcome by the addition of glucose, though not of a mixture of pyruvate and succinate. The special property of glucose not shared by succinate and pyruvate is that it appears to be able to reverse the diminution of the cellular ATP content brought about by deoxyglucose (Table 11).

DISCUSSION

Since the results of Parrish & Kipnis (1964) suggest that insulin can influence amino acid accumulation by muscle only when active transport is involved, the fact that insulin can influence incorporation in the absence of Na^+ (Table 4) emphasizes the point that the influence of insulin on incorporation of amino acids into protein in muscle is not dependent on its effect on amino acid accumulation, even though the two effects may appear to be closely linked (for review see Manchester, 1965). There are now numerous aspects of the metabolism of muscle that show responses to insulin and that so far as can be ascertained respond independently of each other (Manchester, 1965). Whether the insulin effect in each situation starts from a common point is still unknown.

The present results do not provide much support for the concept (Bhattacharya, 1959*a*,*b*, 1961) that Mg^{2+} has a specific function in facilitating the response of muscle to insulin, for the capacity of the tissue to respond to insulin is well maintained under the different conditions studied except (*a*) when the tissue is broken up, (*b*) the ions of the medium are replaced by sucrose and (*c*) when Na^+ is replaced by K^+ and the amino acid is present in a high concentration. One of the aims of the present work was the hope of defining critical factors that determine whether or not a muscle preparation will respond to insulin. The diminution of response to the hormone on cutting the tissue emphasizes the importance of the integrated cell structure for insulin to be able to bring about its action. Whether it is specifically the intact membrane that is necessary for the hormonal response or certain features of the

intracellular cytoskeleton (Peters, 1956; Krahl, 1957) is not known, but clearly the physical state of the tissue has an important bearing on its responsiveness and it is presumably these same considerations that prevent the effects of the hormone in cell-free systems from being seen. It is generally accepted that the capacity of subcellular fragments to incorporate amino acids is much less than that of the intact cell (Hendler, 1962), and this is borne out by the severe diminution in incorporation seen on cutting diaphragm into pieces (Table 9). Hendler (1962) has suggested that protein synthesis near the membrane may be a very significant proportion of the total synthetic activity. If so, physical damage to membranes may be the chief factor involved in the diminution of incorporation when the tissue is cut up.

The loss of insulin response with K^+ buffers might be correlated with the swelling the tissue undergoes (Table 3) and the physical damage to mitochondria and membranes that can be observed under these conditions (Harris, 1961). Incubation in choline buffer, in which swelling does not occur, does not diminish the insulin response (Table 4). Although the results with 2-deoxyglucose might suggest that a decline in the internal ATP content could become a factor limiting the extent of a stimulation of incorporation by insulin, the fact that effects of insulin are readily seen in the choline buffer despite the fall in ATP content (Table 10) argues against the importance of this possibility. Indeed, its interference in the presence of deoxyglucose with an effect of insulin in promoting incorporation is probably a special case arising from the simultaneous stimulation by the hormone of the entry of the sugar into the cell (Kipnis & Cori, 1959), which will aggravate the inhibition of anabolic processes. Normal insulin effects on incorporation expressed as percentage changes can be seen in the presence of metabolic inhibitors or inhibitors of protein synthesis (Manchester, 1961*b*; Wool, Castles & Moyer, 1965).

The decline in the ATP content on the addition of 2-deoxyglucose presumably arises because the sugar can be phosphorylated by diaphragm though not further metabolized (Kipnis & Cori, 1959), and the sugar will thus utilize 'energy-rich' phosphate without leading to its eventual replacement. The presence of deoxyglucose might be expected to promote oxygen consumption, but this does not appear to be so (Woodward & Hudson, 1954; K. L. Manchester, unpublished work). Likewise, the lowered ATP content of diaphragm in all the abnormal buffers (Table 10) may lead to increased oxygen consumption, and this has been noted by Harris (1965) for frog sartorius muscle in choline buffer, but in K^+ medium there did not appear to be much change. If glucose uptake of muscle is inversely related to ATP content (Smith, Randle & Battaglia, 1961), the low ATP content of muscle incubated in sucrose medium would explain the tendency towards high basal glucose uptakes observed by Bhattacharya (1961). Why oxidation of glucose, but not of succinate and pyruvate, can restore towards normal the decline in ATP content in the presence of deoxyglucose is not clear, for both pyruvate and succinate are readily oxidized by diaphragm (Villee, White & Hastings, 1952; K. L. Manchester, unpublished work). DeSchepper *et al.* (1965) showed that the effect of glucose was not attributable to a decrease in the rate of entry of deoxyglucose in the presence of the other sugar as a result of competition for transport. 'Compartmentation' phenomena between cytoplasmic generation of ATP from glucose as opposed to mitochondrial generation from citric acid-cycle intermediates might be of significance since it is uncertain how readily ATP moves into and out of the mitochondrion (Siekevitz & Potter, 1955). Why the addition of pyruvate and succinate promotes any extra oxygen consumption by the tissue at all is problematical, since in the absence of added substrate the basal oxygen consumption of the incubated tissue appears to be adequate to maintain the ATP concentration near its initial level. The addition of substrates to the perfused heart does not influence oxygen consumption (Fisher & Williamson, 1961; Williamson & Krebs, 1961). However, in the presence of pyruvate and succinate, aerobically promoted reduction of the type described by Krebs and co-workers (Kulka, Krebs & Eggleston, 1961; Krebs, Eggleston & d'Alessandro, 1961) might lead to an added requirement for ATP, a factor that would both promote oxygen uptake and be consistent with failure of pyruvate and succinate to raise the ATP content in the presence of deoxyglucose.

The variability of responsiveness to insulin on the substitution of K^+ for Na^+ depending on whether the amino acid is present in a high or low concentration is not easily explained. Although under normal conditions of incubation increasing the concentration of added amino acid diminishes the effects of the hormone on transport phenomena, insulin still observably affects the amount of incorporation even when the amino acid is added at concentrations as high as 20mM (Manchester, 1961*b*; Parrish & Kipnis, 1964). It is not yet known in what way the pool of ^{14}C-labelled amino acids used in protein synthesis is related to the total pool of amino acids in the tissue (Kipnis, Reiss & Helmreich, 1961; Manchester & Wool, 1963). The possibility of a large proportion of the cell's protein synthesis taking place near the membrane would be very germane to this point. If there were a small amino acid pool near the membrane effectively distinct from the

general pool of the tissue, it is possible that with the depolarization and partial destruction of the membrane consequent on substitution of K^+ for Na^+ the action of insulin to influence amino acid accumulation in this area would be less strongly exerted and rendered insignificant more readily as the concentration of added amino acid rose.

The greater diminution in the incorporation of ^{14}C from glycine than from leucine seen in choline buffer (Table 5) is noteworthy in the context of the evidence of Oxender & Christensen (1963) of the factors regulating the entry of amino acids into ascites-tumour cells. They found that the kinetic parameters of the accumulation of amino acids like alanine and glycine were very different from those like leucine and phenylalanine. The former entered the cell more slowly and largely by active accumulation, whereas for the latter group exchange reactions predominated. Qualitatively the characteristics of amino acid accumulation by the perfused rat heart (Manchester & Wool, 1963) seem very similar to those of the ascites-tumour cells and it is probably not unreasonable to extrapolate from these cells to muscle. Thus, if the primary inhibitory influence of Na^+ deprivation is on the active accumulation of amino acids, a more severe diminution of incorporation of glycine than of leucine in the absence of Na^+ is to be expected. A contrast between the behaviour of glycine and of leucine and phenylalanine also shows itself in the comparison of their accumulation and incorporation into protein in the rib-cage preparation and the isolated hemidiaphragm (Manchester & Young, 1960; Table 8). It seems at first sight paradoxical that the active type of accumulation of glycine and aminoisobutyric acid should occur more slowly in the rib-cage preparation than in the isolated hemidiaphragm, for it would be expected that damage to the tissue would interfere with rather than facilitate active accumulation. However, the differences become understandable if the active accumulation of amino acids is linked either to the entry of Na^+ into the cell or to the exit of K^+ (Vidaver, 1964), for both these processes occur to a greater extent in isolated hemidiaphragm than in the rib-cage preparation. The observation of Peckham & Knobil (1962) that crushing diaphragm can under certain conditions enhance the accumulation of aminoisobutyric acid is explicable in a like manner.

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The Effects of Adenine Nucleotides on Carbohydrate Metabolism in Pigeon-Liver Homogenates

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1. The effects of added AMP on carbohydrate metabolism were investigated in pigeon-liver homogenates, which can degrade glucose and synthesize it from lactate. Suitable experimental conditions were established for studying such effects, including the addition of P_i (20mM) to stabilize adenine nucleotides and supplementation with NAD^+ (0·5mM). 2. Lactate increased the rate of oxygen consumption and kept the concentration of ATP high and that of AMP relatively low. 3. Added AMP (1·25–5mM) raised the net rate of carbohydrate removal and inhibited the net formation of glucose from lactate, as well as the incorporation of lactate into glucose. These effects were accompanied by a fall in the concentrations of hexose 6-phosphates and a rise in those of fructose diphosphate and triose phosphates. When the activity of glyceraldehyde 3-phosphate dehydrogenase was limited experimentally by a low concentration of NAD^+ or when it was blocked by iodoacetate, the accumulations of fructose diphosphate and triose phosphates were large and accounted for most of the carbohydrate degraded in the presence of AMP. 4. AMP also inhibited the conversion of pyruvate into phosphoenolpyruvate. Data on the concentrations of pyruvate, phosphoenolpyruvate and intermediates of the tricarboxylic acid cycle, as well as on isotope distribution, suggest that the effect was due to inhibition of phosphoenolpyruvate carboxykinase. 5. The results indicate that in the homogenates phosphofructokinase and fructose diphosphatase, controlled in their activity by adenine nucleotides and other cell constituents, are enzymes which regulate the direction of carbohydrate metabolism (degradation or synthesis) in the liver. 6. It is suggested that active transport of adenine nucleotides, citrate, Mg^{2+}, Ca^{2+}, P_i and other cell constituents may play a role in regulating the activity of enzymes which are affected by these substances. 7. A procedure is described for generating alkali in a closed manometer vessel, by mixing mercuric oxide and a solution of sodium iodide, for use in a method for measuring the oxygen consumption at physiological bicarbonate concentrations.

Apart from being reactants in kinase reactions (hexokinase, glucokinase, phosphofructokinase, phosphoglycerokinase, pyruvate kinase) and in oxidative phosphorylation, adenine 5′-nucleotides are known to affect the activity of at least two key enzymes of hepatic carbohydrate metabolism. Phosphofructokinase is inhibited by ATP and deinhibited by ADP and AMP. Fructose diphosphatase is inhibited by AMP. As these activations and inhibitions are taken to be of importance in the regulation of carbohydrate metabolism (see Krebs, 1964*b*) the effects of AMP on pigeon-liver homogenates, which can degrade carbohydrate by glycolysis and oxidation, and resynthesize it from lactate and other precursors, were investigated. Such homogenates form a convenient system for examining further whether the activations and inhibitions found with isolated enzymes may operate in the multi-enzyme system of the liver cells.

METHODS AND MATERIALS

Materials. The materials were generally as described by Krebs, Dierks & Gascoyne (1964). Special mention must be made of AMP, ADP, ATP, NAD^+ and NADH because of the importance of their purity. AMP was obtained from Waldhof Zellstofffabrik, Mannheim, Germany; ADP was from Sigma Chemical Co., St Louis, Mo., U.S.A.; ATP and NADH, as well as the enzymes required for analyses, were from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Chromatography of AMP and ADP by the

method of Eggleston & Hems (1952) indicated no significant impurities.

Tissue preparations. Homogenates of pigeon liver were prepared as described by Krebs *et al.* (1964), except that the concentrations of P_i and NAD^+ were increased where indicated. To minimize changes in the concentrations of rapidly reacting metabolites during the manipulations after the completion of the incubations, these were terminated by rapid removal of the cups from the water bath, brief immersion, with swirling, in an ethanol–solid CO_2 mixture, transfer to a tray of crushed ice and addition of $HClO_4$ to give a final concentration of 2% (w/v). After 15min. at 0° (sufficient for the extraction of the soluble glycogen) the material was centrifuged at low temperature. A measured sample of the supernatant was neutralized with KOH in the presence of 2 drops of universal indicator (British Drug Houses Ltd., Poole, Dorset) and the precipitate of $KClO_4$ was removed by centrifugation. The supernatant was diluted to a known volume and stored at −15°. Analyses for the adenine nucleotides were carried out within a few hours. The rapid cooling and acid treatment gave higher ATP and lower AMP values than rapid acidification alone.

Measurement of gas exchanges. Since low concentrations of HCO_3^- ions decrease the rate of gluconeogenesis in pigeon-liver homogenates, the O_2 uptake was measured according to the principle of Warburg & Krippahl (1960), which permits the measurement of the O_2 uptake in the presence of physiological concentrations of CO_2 and bicarbonate. The CO_2 pressure is kept approximately constant by the use of 'CO_2 buffers' (Pardee, 1949; Krebs, 1951). Manometer vessels similar to those described by Dickens & Šimer (1931) contained 1ml. of the homogenate in the outer ring and 4ml. of the CO_2 buffer, 25mg. of crude carbonic anhydrase [prepared by the method of Meldrum & Roughton (1933) from bovine erythrocytes] and five small glass beads in the centre compartment. The gas mixture was CO_2+O_2 (5:95). In preparing the CO_2 buffer solution according to the specifications of Warburg & Krippahl (1960) and Warburg, Geissler & Lorenz (1962), it has to be borne in mind that commercial K_2CO_3 invariably contains bicarbonate and moisture and the $KHCO_3$ invariably contains some carbonate. The stock solutions were therefore analysed by titration by the method of Kolthoff & Sandell (1952). A 3M-buffer giving approximately a mixture in equilibrium with 4·2% CO_2 was obtained by dissolving 22·0g. of $KHCO_3$ and 11·2g. of K_2CO_3 (both salts from analytical reagents) in a volume of 100ml. The concentration of bicarbonate should be about 2·28M and that of carbonate 0·72M. The CO_2 pressure above a given buffer mixture was measured by a modification of the principle used by Krebs (1930) for determining the CO_2 content of gas mixtures. HgO (432mg.) and crude carbonic anhydrase (25mg.) were placed in the main compartment of conical Warburg flasks of about 25ml. size fitted with two side arms. Then 1ml. of 4M-NaI was pipetted into one side arm and 1ml. of the buffer into the other immediately before attaching the flask to the manometer. A control vessel contained 1ml. of 4M-NaCl instead of the buffer and 0·2ml. of 2N-NaOH in the centre well. This measured the small gas-pressure changes (less than 5mm.) due to changes in vapour pressure. A thermobarometer contained water. The three manometers were equilibrated briefly at the required temperature. With the taps closed and the level of the meniscus so adjusted as to permit a large increase in gas pressure, the buffer was emptied into the main compartment. This caused a rapid output of CO_2. When equilibrium had been established, the NaI solution was tipped into the main compartment, NaOH was generated by the reaction:

$$2NaI + HgO + H_2O = HgI_2 + 2NaOH$$

and the CO_2 in the gas space was rapidly and quantitatively absorbed. The negative pressure change, corrected for changes in the controls, was the equilibrim CO_2 pressure over the buffer solution. The CO_2 buffers are not perfect absorbers of CO_2 and a correction factor ('retention') has to be measured (see Warburg, 1925; Krebs, 1951; Warburg *et al.* 1962).

To obtain information on the CO_2 production of the homogenates another set was incubated in the standard conical Warburg vessels with CO_2+O_2 (5:95) in the gas space. These vessels contained 4ml. of homogenate. The gas-pressure changes recorded in these vessels represented the sum of the O_2 consumption and the CO_2 formation. These samples also served as the material for the analysis of metabolites.

In a few experiments the O_2 uptake and CO_2 formation were measured by the principle of Warburg & Yabusoe (1924). A phosphate-buffered saline medium with low bicarbonate concentrations (up to 5mM) was used.

Analytical procedures. The methods for the determination of glucose, 'glycogen' (i.e. glucose set free by enzyme hydrolysis), L-malate, citrate, α-oxoglutarate, L-glutamate and L-lactate were the same as those used by Krebs *et al.* (1964). Fumarate was estimated as malate after the addition of fumarase. Glucose 6-phosphate and ATP were estimated by the method of Lamprecht & Trautschold (1963), fructose 6-phosphate by the method of Hohorst (1963*a*), fructose 1,6-diphosphate and combined triose phosphate by the method of Bücher & Hohorst (1963), α-glycerophosphate by the method of Hohorst (1963*b*) and L-aspartate by the method of Pfleiderer (1963). For the determination of the sum of glutamate and glutamine samples were hydrolysed for 2hr. in 2N-H_2SO_4. The analogous hydrolysis of asparagine was carried out in N-HCl.

Pyruvate, ADP and AMP were estimated in a combined procedure based on that described by Adam (1963), but modified to reduce interfering blanks. These were traced to contamination of ATP samples with ADP, and a variable but significant occurrence of AMP in commercial NADH preparations. The former difficulty was overcome by adding ATP (required in the procedure for the adenylate-kinase-catalysed conversion of AMP into ADP) after pyruvate and ADP had been determined, and the second by treating solutions of NADH with a bacterial alkaline phosphatase (Worthington Biochemical Corp., Freehold, N.J., U.S.A.), as suggested by Lowry, Passonneau, Hasselberger & Schulz (1964). The value for ADP obtained includes UDP and GDP, and that for ATP includes ITP. The determination of AMP is specific. Though the quantities of uridine nucleotides in liver tissue are significant in relation to those of adenine nucleotides, this is not the case in dilute homogenates to which large amounts of adenine nucleotides have been added. NAD^+ was determined in the neutral extract by the method of Klingenberg (1963).

Isotopic techniques. [U-^{14}C]Glucose (obtained from The Radiochemical Centre, Amersham, Bucks.) was used at a

specific activity of 0·25 μc/μmole. At the end of incubation, 100 μl. samples of the final neutral extracts were spotted on Whatman no. 1 paper and descending chromatograms in two directions were carried out at room temperature by using the 'semi-stench' of Crowley, Moses & Ullrich (1962) and butan-1-ol–propionic acid–water (47:22:31, by vol.) as solvents. After being dried, the papers were placed on Kodirex X-ray film (Kodak Ltd., London, W.C. 2) for 3 weeks. A further series of 0·4 ml. samples were added to 'diazyme' (Takamine; Miles Chemical Co., Clifton, N.J., U.S.A.; 0·2% in 0·01 M-acetate buffer, pH 4·4). These were incubated at 50° for 2 hr. and then heated in a boiling-water bath for 5 min. After centrifugation 200 μl. samples of the supernatants were chromatographed. Spots were identified by co-chromatography with glucose. The radioactivity of the glucose spots was determined directly on the paper with an end-window Geiger–Müller tube and a Panax scaler (Panax Equipment Ltd., Redhill, Surrey). Both sides of the paper were counted to a minimum of 10000 counts, corrected for background activity, and the mean was taken to be the number of counts/min. in the spot; differences between the two sides were found to be less than 1%. The amount of glucose in the spots proved to be identical with that in the original extracts before chromatography.

The radioactivity of $^{14}CO_2$ formed was measured as follows. The manometer was connected with rubber tubing to a suction system and the CO_2 of the gas phase and medium, including the bicarbonate, was collected (Sakami, 1955). As described by Pinter, Hamilton & Miller (1963), 10 μl. of the alkali was spotted on a small square of glass-fibre filter paper (Whatman GF/A; H. Reeve Angel and Co. Ltd., London, E.C. 4) and placed in a glass vial for liquid-scintillation counting. The sample was dried at 110° for 10 min. and, after cooling, 10 ml. of a scintillation solution [700 ml. of A.R. toluene, 300 ml. of ethanol, 3 g. of 2,5-diphenyloxazole and 0·1 g. of 1,4-bis-(5-phenyloxazol-2-yl)benzene] was added. The samples were counted in a liquid-scintillation counter (Isotope Developments Ltd., Beenham, Reading, Berks.) and the final values corrected for background activity. The radioactivity of the glucose used in this experiment was determined by the same procedure. The radioactivity was not affected by the addition of 2 N-NaOH to the sample on the glass-paper.

RESULTS

General properties of pigeon-liver homogenates

Stability of nucleotides. Preliminary experiments indicated that added adenine nucleotides rapidly disappear in pigeon-liver homogenates prepared by the method of Krebs *et al.* (1964). Most of the added nucleotides was lost within 10–20 min. at 40°, the degradation being mainly due to 5′-AMP deaminase and 5′-nucleotidase (Dresel, 1953; see also Haugaard, Inesi & Blanken, 1960; Tiedemann & Born, 1963; Berry, 1965). Both these enzymes are known to be inhibited by P_i (Kizer, Cox, Lovig & de Estrugo, 1963; Segal & Brenner, 1960), and, as expected, increasing the P_i concentration in the incubation medium from 4·7 to 20 mM greatly diminished the loss of nucleotide, especially in the presence of lactate (Table 1). The results shown in Table 1 indicate that the protective effect of lactate at 20 mM-P_i is associated with the maintenance of a high concentration of ATP and a low concentration of AMP, the adenine nucleotide most liable to enzymic hydrolysis. Because of its stabilizing effect, 20 mM-P_i was used in most subsequent experiments. Lactate increases the rate of respiration (Krebs *et al.* 1964), and the maintenance of the ATP concentration is thus probably due to the greater effective-

Table 1. *Effect of the concentration of P_i on the stability of adenine nucleotides in pigeon-liver homogenates*

The data refer to 4 ml. of pigeon-liver homogenate prepared and incubated as described in the text. The pigeons were starved for 48 hr. Glucose (5 mM) was added in all experiments. AMP was added as a 0·1 M solution of the disodium salt from the side arm after 20 min. and incubation was continued for another 20 min. The reaction was stopped by rapid cooling (see the text) and the addition of $HClO_4$. 'Preformed' adenine nucleotides refers to the sum of AMP, ADP and ATP found at zero time.

Expt. no.	Concn. of P_i (mM)	Concn. of added lactate (mM)	Added AMP (μmoles/cup)	Preformed adenine nucleotides (μmole/cup)	Adenine nucleotides found at end of incubation (μmoles/cup) ATP	ADP	AMP	Total	Change of nucleotides (μmoles/cup)	$\frac{[AMP][ATP]}{[ADP]^2}$
1	1	0	10	0·86	0·11	0·55	1·26	1·91	−8·94	0·46
	1	40	10	0·86	0·43	0·88	0·82	2·13	−8·73	0·46
2	4·7	0	20	0·66	0·49	0·63	0·44	1·56	−19·1	0·54
	4·7	40	20	0·66	0·82	0·45	0·13	1·40	−19·3	0·53
3	20	0	10	0·87	3·98	2·52	0·81	7·31	−3·56	0·51
	20	40	10	0·87	8·91	1·68	0·20	10·8	−0·07	0·63
	20	0	20	0·87	2·32	3·58	3·50	9·40	−11·5	0·63
	20	40	20	0·87	12·9	2·38	0·27	15·6	−5·2	0·61

ness of coupled oxidative phosphorylation in the presence of lactate.

NAD^+ rapidly disappears during the preparation of the homogenate (see Krebs & Hems, 1964) and on incubation both endogenous and added NAD^+ were extensively degraded under standard conditions. Nicotinamide at 20mM inhibited this process. When NAD^+ (0·5mM) was added to the medium, almost half was lost in 45min. in the absence of nicotinamide, and about 20% with 20mM-nicotinamide. The addition of AMP (2·5mM) did not affect the rate of NAD^+ loss nor was this affected by increasing the P_i concentration from 5 to 20 mM.

Equilibrium in the adenylate-kinase system. The value of the quotient $[AMP][ATP]/[ADP]^2$, as calculated from the data obtained in these experiments, varied between 0·45 and 0·65, apart from instances where low values for AMP introduced a large error. The equilibrium constant varies between 0·4 and 0·9 with the Mg^{2+} concentration (H. A. Krebs, W. Gevers & T. Gascoyne, unpublished work; see also Bowen & Kerwin, 1956). Thus adenylate kinase maintained the concentrations of the reactants near the equilibrium position. This reflects the high activity of this enzyme in liver (Oliver, 1955) compared with the rate of ATP synthesis and utilization.

Effects of added AMP on homogenates of liver from starved pigeons, in the absence and presence of lactate

Effects of the addition of AMP on the formation and disappearance of carbohydrate. Glucose was added (5mM) to the homogenates because, unlike those from well-fed birds, livers from starved pigeons contained insufficient carbohydrate to saturate the capacity of the homogenate to metabolize glucose. Added glucose disappeared on incubation and this was greatly accelerated by AMP (Table 2, cups 2 and 3). The formation of glycogen did not account for the removal of glucose. The addition of lactate plus glucose gave rise to substantial gluconeogenesis (cup 5), which was completely abolished by AMP (cup 6). In fact, in this experiment more glucose disappeared in the presence than in the absence of lactate.

Similar effects of AMP were observed in over 20 other experiments. Final AMP concentrations of 1·25mM were generally more effective than concentrations of 5mM. At mM-P_i, the effect of AMP on glucose disappearance was smaller.

Effects of AMP on the concentration of intermediates of carbohydrate metabolism. The addition of AMP caused characteristic changes in the concentrations of intermediary metabolites (Table 3), which depended on the presence of lactate. When no AMP was added the concentrations of glucose 6-phosphate and fructose 6-phosphate increased on incubation, and these increases were approximately doubled by lactate. The addition of AMP (final concentrations 0·1–2mM) led to a fall in the concentrations of both hexose phosphates and abolished the increases found on incubation with lactate.

The most striking changes were those in the concentrations of fructose diphosphate and triose phosphates, which increased more than 100-fold on the addition of AMP in the absence of lactate. There was a relatively small increase without AMP. Lactate abolished these increases and, instead of fructose diphosphate and triose phosphates, large quantities of α-glycerophosphate appeared, especially when AMP was added.

Table 2. *Effects of added AMP on carbohydrate metabolism in pigeon-liver homogenate (bird starved for 48hr.)*

The data refer to 4ml. of liver homogenate. The medium contained P_i (20mM) and $NaHCO_3$ (25mM); the gas space contained CO_2+O_2 (5:95). Glucose (5mM) was added. AMP was added from a side arm after 15min. and the incubation was continued for another 30min. The tissue dry wt. was 80mg./cup.

Cup no.	Initial	1	2	3	4	5	6
Additions (other than glucose):							
Lactate (mM)					40	40	40
AMP (mM)				2·5			2·5
Time of incubation (min.)	0	15	45	45	15	45	45
Metabolites found (μmoles/cup)							
Glucose	2·5	19·5	14·2	5·9	23·9	27·5	6·6
Glucose+glycogen	3·9	21·1	16·8	8·5	25·5	30·5	8·4
Glycogen	1·4	1·6	2·6	2·6	1·6	3·0	1·8
Changes between 15 and 45min. (μmoles/cup)							
Glucose			−5·3	−13·6		+3·6	−17·3
Glucose+glycogen			−4·3	−12·6		+5·0	−17·1
Glycogen			+1·0	+1·0		+1·5	+0·3

Table 3. *Effects of AMP on the concentration of intermediates of carbohydrate metabolism in pigeon-liver homogenate (starved bird)*

The data refer to the same experiment for which values for glucose and glycogen are given in Table 2.

Cup no.	Initial	1	2	3	4	5	6
Additions other than glucose		None	None	AMP	Lactate	Lactate	Lactate + AMP
Time of incubation (min.)	0	15	45	45	15	45	45
Intermediates found (μmoles/cup)							
Glucose 6-phosphate	0·13	0·74	0·63	0·18	1·39	1·10	0·59
Fructose 6-phosphate	0·04	0·20	0·15	0·04	0·40	0·30	0·10
Fructose 1,6-diphosphate	0·02	0·02	0·12	8·46	0·03	0·03	0·13
Triose phosphates	0·08	0·11	0·41	9·71	0·12	0·14	0·42
α-Glycerophosphate	0·26	0·18	0·58	0·90	8·18	4·28	15·1
3-Phosphoglycerate	0·20	0·16	0·14	0·08	1·10	1·24	0·28
2-Phosphoglycerate	0·06	0·06	0·05	0·02	0·16	0·17	0·04
Phosphoenolpyruvate	0·12	0·09	0·09	0·03	0·55	0·66	0·10
Pyruvate	0·10	0·07	0·10	0·11	0·37	0·38	0·27
Lactate	1·47	0·23	0·80	0·81	140	97·5	89·0
Changes in lactate (μmoles/cup)	—	−1·24	−0·67	−0·68	−21·5	−64·0	−72·5

The accumulation of fructose diphosphate and triose phosphates did not occur in homogenates from starved birds to which no glucose was added; in other words, the presence of glucose or glycogen was a prerequisite of the accumulation. This suggests that the bulk of the three intermediates was derived from carbohydrate rather than from gluconeogenic precursors. Results similar to those in Table 3 were obtained in every experiment where (1) the AMP concentration measured at the end of incubation was 0·1mM or higher, (2) the concentration of added NAD^+ was 0·125mM or less, (3) glucose was added and (4) the concentration of P_i was 5mM or more. Greater amounts of 3-phosphoglycerate, 2-phosphoglycerate, phosphoenolpyruvate and pyruvate were found at the end of the incubation in the presence than in the absence of lactate, but AMP caused a fall in the concentrations of all four intermediates with and without lactate. This fall was greatest for phosphoenolpyruvate and least for pyruvate.

These observations are all in agreement with the assumption that rate-limiting reactions and the factors controlling the rates in the homogenates are the following (only activations and inhibitions relevant to the interpretation of the observations are listed): (1) phosphofructokinase, activated by AMP and ADP (Passonneau & Lowry, 1964; Underwood & Newsholme, 1965*b*); (2) hexokinase, inhibited by glucose 6-phosphate (Weil-Malherbe & Bone, 1951; Crane & Sols, 1954); (3) fructose diphosphatase, inhibited by AMP and by fructose diphosphate above 0·1mM (Taketa & Pogell, 1963; Underwood & Newsholme, 1965*a*); (4) triose phosphate dehydrogenase, limited in the direction of glycolysis by a low concentration of NAD^+ far below the K_m value of the enzyme for NAD^+, but almost fully active in the reverse direction (assuming that the K_m values are of the same order as for rabbit-muscle triose phosphate dehydrogenase, where K_m for NAD at pH 7·4 and 26° is 100μM and that for NADH is 3·3μM; Velick & Furfine, 1963).

The three changes that occur on the addition of AMP, namely the rise in the concentrations of fructose diphosphate and triose phosphates, the fall in those of hexose 6-phosphates and the increased removal of glucose, can be accounted for by the behaviour of phosphofructokinase (effect 1), plus the increased activity of hexokinase (effect 2). The inhibition of fructose diphosphatase by AMP and fructose diphosphate (effect 3) accounts for the flow of carbohydrate metabolism in the direction of glycolysis as opposed to gluconeogenesis. That glycolysis does not proceed beyond the formation of triose phosphate and causes an accumulation of fructose diphosphate and triose phosphates is due to effect 4.

The changes that occur in the presence of lactate and absence of added AMP, namely a fall in the concentration of fructose diphosphate and rise in those of hexose 6-phosphates, are accounted for by the de-inhibition of fructose diphosphatase (effect 3). The occurrence of a net synthesis of carbohydrate shows that triose phosphate dehydrogenase is active in the reduction of 1,3-diphosphoglycerate (effect 4), as expected on account of the measured concentration of NADH in the homogenate (6–10μM; Krebs & Hems, 1964). The rise in concentrations of the phosphoglycerates and phosphoenolpyruvate in the presence of lactate suggests that the formation of these intermediates is more rapid than their removal.

Table 4. *Effect of NAD⁺ concentration on carbohydrate utilization in pigeon-liver homogenate (starved bird)*

Each cup contained 4ml. of homogenate (dry wt. 69mg.) prepared as described in the Methods and Materials section and incubated for 45min. AMP was added at 15min. The homogenate contained initially (μmoles/cup): glucose plus glycogen, 3·2; glucose, 1·8; fructose diphosphate, 0·03; triose phosphates, 0·13; α-glycerophosphate, 0·37.

Cup no.	1	2	3	4
Additions:				
Glucose (mM)	5	5	5	5
NAD^+ (mM)			0·5	0·5
AMP (mM)		2·5		2·5
Changes in compounds (μmoles/cup)				
Glucose+glycogen	−6·8	−11·4	−8·5	−13·3
Glucose	−9·0	−13·2	−0·4	−14·7
Fructose diphosphate	+0·44	+4·61	+0·04	+0·13
Triose phosphates	+1·71	+6·87	+0·05	+0·16
α-Glycerophosphate	+0·07	+0·31	−0·20	−0·09

Effects of NAD^+ concentration in the absence of lactate. Adding 4 times as much NAD^+ (0·5mM) to the homogenate as in previous experiments increased the disappearance of carbohydrate on incubation. The addition of AMP (2·5mM) further accelerated the removal (Table 4). At this higher NAD^+ concentration the addition of AMP caused no accumulation of fructose diphosphate and triose phosphates. The observations are accounted for by the rate-limiting function of NAD^+ in the triose phosphate-dehydrogenase system (effect 4). At 0·5mM-NAD^+ this step no longer blocks the degradation of triose phosphate. Hence, in the absence of lactate, more glucose is broken to the stages of pyruvate and beyond, and this breakdown is greatly enhanced by AMP (effects 1, 2 and 3). This interpretation is confirmed by another experiment in which the yield of $^{14}CO_2$ from [U-^{14}C]-glucose (2·5mM) was determined during a 30min. incubation after the addition of AMP (2·5mM) in the absence of lactate. AMP caused a 2·5-fold increase in the number of counts in carbon dioxide.

Effect of NAD^+ concentration in the presence of lactate. The synthesis of carbohydrate in the presence of lactate was not affected by the addition of NAD^+ (0·5mM) (effect 4). A detailed analysis of the effects of AMP in such homogenates to which lactate and [U-^{14}C]glucose were added, and where gluconeogenesis predominated over glycolysis, was made in a further experiment (Table 5). The synthesis of carbohydrate that occurred in the absence of AMP was associated with a progressive fall in the specific activity both of glucose and of glucose plus glycogen until, at 90min., it was less than half the starting value. However, only about one-fifth of the total radioactivity was not recovered as glucose or glycogen, which indicates a lower rate of glucose degradation under the test conditions (i.e. in the presence of lactate). In contrast, there was a net disappearance of glucose in the early stages after the addition of AMP, whereas the concentration of AMP, which fell (cup 3) from 2·5 to 0·23mM in 15min., remained high enough to inhibit. When the concentration of AMP dropped further to 0·14mM after 60min., a net synthesis of glucose began. There was no fall in the specific activity of glucose until net synthesis occurred. The data for the radioactivity of glucose plus glycogen were similar to those found for free glucose; hence the fall in specific activity was not due to the formation of glucose from glycogen.

The concentrations of intermediates in these experiments were very similar to those given in Table 3, describing an experiment where the concentration of added NAD^+ was 0·125mM, except that the concentration of fructose diphosphate at no stage rose above 0·01mM (Table 5). Adding NAD^+ (0·5mM) did not abolish the extra formation of α-glycerophosphate caused by the addition of AMP in the presence of high concentrations of lactate. The amount of radioactivity in α-glycerophosphate increased sharply (Table 5). During the first 15min. after AMP addition, 41900 counts/min. were lost from glucose, of which 33700 counts/min. appeared in α-glycerophosphate; the net increase in the latter was 5·12μmoles/cup, which would, if derived entirely from glucose, account for 31600 counts/min. Thus AMP, on balance, caused a breakdown of carbohydrate to the level of triose phosphate; but, in the presence of lactate, triose phosphate was not dehydrogenated (effect 4) but reduced to α-glycerophosphate in accordance with the reaction:

Lactate + dihydroxyacetone phosphate
= pyruvate + α-glycerophosphate

Table 5. *Effects of AMP on the synthesis and turnover of carbohydrate in pigeon-liver homogenate (starved bird)*

Each cup contained 4 ml. of homogenate incubated for the periods given in the Table. Each cup contained 2·5 μc of [U-^{14}C]glucose, and the medium contained P_i (20 mM) and NAD^+ (0·5 mM). AMP was added to the relevant cups at 15 min. Other procedures are described in detail in the text. The tissue dry wt. was 74·2 mg./cup.

Cup no.	Initial	1	2	3	4	5	6	7	8	9
Time (min.)	0	15	30	30	45	45	60	60	60	60
Additions:										
Glucose (mM)	2·5	2·5	2·5	2·5	2·5	2·5	2·5	2·5	2·5	2·5
Lactate (mM)	35	35	35	35	35	35	35	35	35	35
AMP (mM)				2·5		2·5		2·5		2·5
Compounds found										
Glucose + glycogen:										
μmoles/cup	7·4	18·2	21·5	14·5	24·6	14·9	27·5	15·7	29·8	22·7
Counts/min./cup ($\times 10^3$)	211	201	197	158	191	144	176	140	170	149
Counts/min./μmole ($\times 10^2$)	121	110	92	109	78	97	64	89	57	65
Free glucose:										
μmoles/cup	4·4	16·1	19·5	12·7	20·6	11·9	22·5	12·9	21·3	18·8
Counts/min./cup ($\times 10^3$)	211	199	193	157	180	141	161	137	149	139
Counts/min./μmole ($\times 10^2$)	146	123	99	123	87	118	72	106	70	74
Glycogen:										
μmoles/cup	3·0	2·1	2·0	1·8	4·0	3·0	5·0	2·8	8·5	3·9
Counts/min./cup ($\times 10^3$)	0	2	4	1	11	3	15	3	21	10
Counts/min./μmole ($\times 10^2$)	0	10	20	6	28	10	30	11	24	26
α-Glycerophosphate:										
μmoles/cup	0·64	7·53	6·79	12·6	4·08	13·4	2·56	12·9	1·61	6·83
Counts/min./cup ($\times 10^3$)	0	5·1	6·25	38·8	6·15	33·2	3·9	27·1	1·4	13·5
Counts/min./μmole ($\times 10^2$)	0	6·8	9·2	30·8	15·1	24·7	15·2	21·0	8·7	19·8
Fructose diphosphate:										
μmoles/cup	0·04	0·01	0·03	0·03	0·03	0·03	0·01	0·03	0·01	0·01
AMP:										
μmoles/cup	0·48	0·25	0·37	0·92	0·32	0·68	0·29	0·56	0·33	0·47

Table 6. *Equilibrium between two linked dehydrogenase systems in pigeon-liver homogenate*

The values given refer to the experiment described in Table 5. Determinations were carried out as indicated in the Methods and Materials section. The value for dihydroxyacetone phosphate was calculated from that for the sum of triose phosphates, assuming that there was equilibrium in the triose phosphate-isomerase system and that K=[dihydroxyacetone phosphate]/[glyceraldehyde 3-phosphate]=22 (Baronowski, 1949). E_0' values of the lactate–pyruvate and α-glycerophosphate–dihydroxyacetone phosphate couples at 25° were taken to be −0·185 and −0·192 v respectively (Burton, 1957), and the expected ratio K=[α-glycerophosphate][pyruvate]/[lactate][dihydroxyacetone phosphate]=0·58 was derived from the relation $\Delta E_0'=0{\cdot}03 \log K$.

			Compounds found (μmoles/cup)				
Cup no.	Time (min.)	Added AMP (μmoles/cup)	Lactate	Dihydroxy-acetone phosphate	Pyruvate	α-Glycero-phosphate	K
1	15	0	124	0·16	1·51	7·53	0·57
2	30	0	115	0·19	1·81	6·79	0·56
3	30	10	101	0·32	1·08	12·6	0·42
4	45	0	88·9	0·15	1·86	4·08	0·57
5	45	10	79·0	0·34	0·93	13·4	0·47
6	60	0	75·3	0·14	1·84	2·56	0·45
7	60	10	63·1	0·29	0·85	12·9	0·60
8	90	0	54·0	0·15	0·72	1·61	0·14
9	90	10	35·6	0·24	0·84	6·83	0·67

Table 7. *Effects of AMP on the formation of intermediates of the tricarboxylic acid cycle and related amino acids in pigeon-liver homogenates in the presence of lactate (starved bird)*

Each cup contained 4 ml. of homogenate prepared as described in the Methods and Materials section and incubation was for 60 min. The medium contained P_i (20 mM) and NAD^+ (0·5 mM). AMP was present in cup 2 from zero time. The calculation of 'lactate equivalents' was made according to the principles described by Berry (1965) for the calculation of 'pyruvate equivalents' of intermediates measured. The tissue dry wt. was 80·4 mg./cup.

Cup no....	Initial	1	2
Additions:			
Lactate (mM) ...		40	40
AMP (mM)			2·5
Intermediates found (μmoles/cup)			
Glutamate	0·97	2·11	2·66
Glutamine	0·20	0·46	0·37
Aspartate	0·71	0·87	2·09
Asparagine	0·05	0·01	0·02
Alanine	0·85	2·34	3·52
Citrate	0·49	1·23	1·86
α-Oxoglutarate	0·14	3·26	2·94
Fumarate	<0·01	1·10	1·99
Malate	0·62	6·75	12·2
Lactate removed (μmoles/cup)		−57·6	−72·2
Total 'lactate equivalents' gained (μmoles/cup)		+21·2	+31·5

The concentrations of the four reactants were close to those of the equilibrium state (Table 6).

At the same time, AMP inhibited the initial stages of gluconeogenesis from lactate to triose phosphate, as indicated by the fact that lactate carbon was not incorporated into glucose or α-glycerophosphate. This inhibition was not due to decreased utilization of lactate because the data in Table 7 show that more lactate was removed when AMP was added. The conclusion must be drawn that AMP above a critical concentration causes pyruvate to undergo oxidation rather than conversion into phosphoenolpyruvate. This effect could be exerted either by stimulation of the rate of pyruvate oxidation or by the inhibition of an enzyme concerned with the synthesis of phosphoenolpyruvate.

Evidence in favour of the first possibility is: (*a*) the increase in the rate of oxygen uptake, and increased oxidation of lactate to carbon dioxide, caused by AMP (see below), presumably related to the formation of ADP by the action of adenylate kinase; (*b*) the fall in the steady-state concentration of pyruvate to about half that found in the absence of AMP (Table 6).

Evidence in favour of the second possibility is: (*a*) the fall in the concentration of phosphoenolpyruvate (and in that of the phosphoglycerates) caused by AMP (Table 2), which could be due to diminished activity of phosphoenolpyruvate carboxykinase or to enhanced action of pyruvate kinase; (*b*) the rise in the concentration of malate to nearly double that found when AMP was not added, in conjunction with the increased rate of formation of other intermediates of the tricarboxylic acid cycle and related amino acids (Table 7). This suggests that pyruvate carboxylase is not

affected by AMP or by the products of its metabolism. However, an inhibition of phosphoenolpyruvate carboxykinase, the second enzyme responsible in avian liver for the conversion of pyruvate into phosphoenolpyruvate, could account for the effects of AMP, a point that requires further investigation. It is noteworthy that Holten & Nordlie (1965) have in fact observed an inhibition of guinea-pig-liver mitochondrial phosphoenolpyruvate carboxykinase by AMP.

Effect of AMP on gas exchanges of pigeon-liver homogenate. The addition of glucose alone caused a small increase in the oxygen consumption, and a further rise, under optimum conditions, of about 50% was brought about by AMP (Table 8). Optimum conditions included the presence of 20mM-P_i and 5mM-bicarbonate or more in the medium: the effect was slight when the concentration of P_i was 1mM, and without bicarbonate there was no stimulation by AMP. Lactate alone also caused a great increase in oxygen uptake (about 100%) and the extra rise due to AMP was only 20–30%. Varying the concentration of added AMP from 1·25 to 5mM made little difference. At 2·5mM the rise in oxygen uptake was progressive with time (Table 9); at 5mM initial stimulation was followed by inhibition when the concentration of P_i was 5mM, and in the latter case extensive losses of adenine nucleotides occurred during the incubation. The inhibition was probably due to an effect of ammonia set free from AMP (see Recknagel & Potter, 1951; Berry, 1964). The respiratory quotient was 0·85 under standard conditions (Table 8, Expt. 1) and the addition of AMP did not change this value despite the large increases in the consumption of both oxygen and carbohydrate.

In homogenates containing very small initial quantities of carbohydrate and to which no glucose

Table 8. *Effects of AMP on gas exchange in pigeon-liver homogenate (starved bird)*

In each experiment, 4ml. of homogenate was incubated for 45min.; full details of procedure are given in the Methods and Materials section. The P_i concentration was 20mM. Experiments were carried out with and without AMP (5mM in Expt. 1 and 2·5mM in the others), which was added from a side arm at 15min. CO_2 buffers were used in Expt. 1 and the method of Warburg & Yabusoe (1924) in Expts. 2–5. The gas measurements refer to the periods 15–45min. The tissue dry wt. varied from 70 to 80mg. Expt. 1 is the same as that described in Tables 2 and 3, where values for changes in carbohydrates and intermediates are given. R.Q., Respiratory quotient.

Expt. no.	Substrate added	Concn. of substrate (mM)	Concn. of added NAD^+ (mM)	Concn. of HCO_3^- (mM)	Without AMP		With AMP		Increase of O_2 uptake caused by AMP (%)
					O_2 uptake (μmoles/cup)	R.Q.	O_2 uptake (μmoles/cup)	R.Q.	
1	Glucose	5	0·125	25	−39·8	0·85	−61·3	0·85	54
	Glucose, lactate	5, 40	0·125	25	−56·4	0·55	−72·0	0·59	27
2	Glucose	5	0·125	0	−32·0	0·84	−33·4	0·85	4
3	Glucose	5	0·125	5	−34·1	0·91	−50·8	0·90	49
4	Glucose	5	0·5	5	−33·5	0·85	−42·2	0·95	26
5	None		0·125	5	−25·2	0·89	−30·9	0·88	23

Table 9. *Time-course of AMP effect on oxygen uptake by pigeon-liver homogenate*

Conditions were as described in Table 8 and Expt. 1 corresponds to Expt. 1 of that Table. CO_2 buffers were used to measure the uptake of O_2. AMP was added at 15min. The tissue dry wt. was 80mg./cup in the first experiment and 75mg./cup in the second.

Expt. no.	Substrate added	Concn. of substrate (mM)	Concn. of P_i (mM)	Concn. of added AMP (mM)	O_2 uptake (μmoles/cup)			Total loss of adenine nucleotides during incubation (μmoles/cup)
					0–15min.	15–30min.	30–45min.	
1	Glucose	5	20	0	−20·8	−20·2	−19·6	—
	Glucose	5	20	2·5	−20·7	−27·8	−33·5	−3·4
	Glucose, lactate	5, 40	20	0	−32·2	−30·3	−26·1	—
	Glucose, lactate	5, 40	20	2·5	−32·2	−33·5	−38·5	−0·1
2	Glucose	5	5	0	−22·1	−21·5	−21·1	—
	Glucose	5	5	5	−22·0	−27·4	−11·2	−18·4

was added the increase in oxygen uptake brought about by AMP was only 23% (Table 8, Expt. 5). Virtually all the preformed glucose and glycogen disappeared on incubation whether AMP was added or not.

Effects of added AMP on liver homogenates from well-fed pigeons

Effects of AMP on the formation and degradation of carbohydrate. In homogenates of liver from well-fed pigeons, AMP caused an increase in the rate of disappearance of total carbohydrate, mainly glucose (Table 10). When lactate was added, some gluconeogenesis occurred, but as described by Krebs *et al.* (1964) the rate was low. When AMP and lactate were added together no net synthesis of carbohydrate occurred but much carbohydrate disappeared. The absolute effect of AMP when corrected for the control without AMP was about the same without lactate (cups 2 and 3: 10·3 μmoles) and with lactate (cups 5 and 6: 11·1 μmoles).

Effects of AMP on the concentration of intermediates. The effects of AMP on the concentrations of intermediates were similar to those found in homogenates from starved pigeons (Table 11). When NAD^+ was added at 0·5 mM, fructose diphosphate and triose phosphates did not accumulate. Iodoacetate at 0·25 mM (in the absence of lactate) and NAD^+ added at 0·5 mM (Table 12) prevented the

Table 10. *Effects of added AMP on carbohydrate metabolism in pigeon-liver homogenate (well-fed bird)*

Conditions were as described in Table 2. The tissue dry wt. was 80 mg./cup.

Cup no.	Initial	1	2	3	4	5	6
Additions:							
Lactate (mM)					40	40	40
AMP (mM)				2·5			2·5
Time of incubation (min.)	0	15	45	45	15	45	45
Metabolites found (μmoles/cup):							
Glucose	12·2	20·8	15·9	9·4	19·6	18·8	15·0
Glucose + glycogen	71·2	71·5	70·3	60·0	70·5	74·8	63·7
Glycogen	59·0	50·7	54·4	50·6	50·9	56·0	48·7
Changes between 15 and 45 min. (μmoles/cup):							
Glucose			−4·9	−11·4		−0·8	−4·6
Glucose + glycogen			−1·2	−11·5		+4·3	−6·8
Glycogen			+3·7	−0·1		+5·1	−2·2

Table 11. *Effects of AMP on the concentration of intermediates of carbohydrate metabolism in pigeon-liver homogenate (well-fed bird)*

The values refer to the experiment described in Table 10, where conditions of incubation are given.

Cup no.	Initial	1	2	3	4	5	6
Additions		None	None	AMP	Lactate	Lactate	AMP + lactate
Time of incubation (min.)	0	15	45	45	15	45	45
Intermediates found (μmoles/cup)							
Glucose 6-phosphate	0·78	0·79	0·75	0·52	1·08	1·20	0·86
Fructose 6-phosphate	0·26	0·25	0·24	0·18	0·35	0·39	0·30
Fructose diphosphate	0·13	0·25	0·55	5·11	0·12	0·11	0·14
Triose phosphates	0·26	0·65	1·62	8·58	0·21	0·17	0·28
α-Glycerophosphate	1·66	0·51	0·27	0·58	5·12	7·86	14·5
3-Phosphoglycerate	0·32	0·30	0·30	0·14	0·45	0·49	0·21
2-Phosphoglycerate	0·07	0·06	0·05	0·03	0·06	0·08	0·04
Phosphoenolpyruvate	0·14	0·13	0·14	0·06	0·28	0·26	0·15
Pyruvate	0·10	0·10	0·09	0·04	0·35	0·38	0·19
Lactate	2·45	1·10	0·52	0·71	149·1	124·0	119·5
Change in lactate (μmoles/cup)		−1·35	−1·93	−1·74	−14·8	−39·9	−44·4

Table 12. *Effect of iodoacetate and AMP on pigeon-liver homogenate (well-fed bird)*

Each cup contained 4ml. of homogenate. AMP was added at 15min. during the incubation of 45min.; iodoacetate was present initially. The tissue dry wt. was 83mg./cup.

Cup. no.	Initial	1	2	3	4
Additions:					
NAD^+ (mM)		0·5	0·5	0·5	0·5
Sodium iodoacetate (mM)...				0·25	0·25
AMP (mM)			2·5		2·5
Intermediates found (μmoles/cup)					
Glucose+glycogen	33·7	25·2	20·2	33·2	22·8
Glucose	8·45	5·98	4·23	13·5	10·0
Glycogen	25·2	19·2	16·0	19·7	12·8
Fructose diphosphate	0·06	0·07	0·10	0·09	7·80
Triose phosphates	0·19	0·26	0·32	0·47	10·1
α-Glycerophosphate	1·39	0·31	0·54	1·02	1·19

Table 13. *Effects of 2,4-dinitrophenol on carbohydrate synthesis in pigeon-liver homogenate (starved bird)*

Each cup contained 4ml. of homogenate incubated for 45min. The medium contained lactate (40mM) but no added glucose; the concentration of P_i was 20mM. The tissue dry wt. was 74·1mg./cup.

Cup. no.	1	2	3	4
Dinitrophenol (mM)... ...	0	0·02	0·05	0·1
O_2 uptake (μmoles/cup) ...	−70·4	−72·3	−70·5	−68·8
Metabolites found (μmoles/cup)				
Glucose	13·9	13·2	7·17	2·68
Glucose+glycogen	18·1	15·7	10·1	5·18
Glucose 6-phosphate	0·88	0·80	0·59	0·28
Fructose 6-phosphate	0·22	0·22	0·14	0·05
Fructose 1,6-diphosphate	0·03	0·03	0·03	0·05
Triose phosphates	0·12	0·14	0·12	0·23
α-Glycerophosphate	2·78	4·02	3·63	4·42
Pyruvate	0·77	0·36	0·29	0·25
ATP	0·97	0·90	0·84	0·37
ADP	0·32	0·38	0·50	0·71
AMP	0·05	0·10	0·15	0·46

removal of total carbohydrate during incubation (although breakdown of glycogen to glucose occurred), and the concentrations of intermediates did not increase significantly. When AMP was added, however, a large amount of carbohydrate disappeared: this could be accounted for quantitatively as fructose diphosphate and triose phosphates. This confirms the conclusions reached above that the primary effects of AMP were on phosphofructokinase and fructose diphosphatase (effects 1 and 2).

Effects of 2,4-dinitrophenol. Experiments with dinitrophenol were carried out to ascertain whether interference with the maintenance of ATP synthesis would simulate the effects of AMP addition in a specific way. The ATP concentration fell slightly at 0·05mM-dinitrophenol, and by about 60% at 0·1mM-dinitrophenol (Table 13). There were corresponding rises in the concentrations of AMP and ADP. These changes of the adenine nucleotides were paralleled by a fall in carbohydrate synthesis. Though there was no accumulation of fructose diphosphate or triose phosphates, the concentration of glucose 6-phosphate was decreased and that of α-glycerophosphate increased. It is thus feasible that the effects of dinitrophenol on carbohydrate metabolism were due to the rise in the concentration of AMP.

Effects of the addition of ADP and ATP. The addition of ADP or ATP caused changes similar to those obtained with AMP. This may be expected, owing to the ready interconversion of the adenine nucleotides. No evidence was obtained to indicate that ATP has specific effects, except that, in homogenates of well-fed bird livers with high initial glycogen content, high concentrations (5mM) of

ATP caused rapid glycogenolysis with increases in free glucose and glucose 6-phosphate; this was possibly due to activation of glycogen phosphorylase by 3′,5′-(cyclic)-AMP formed from ATP.

DISCUSSION

Control of phosphofructokinase and fructose diphosphatase by adenine nucleotides. The results obtained with pigeon-liver homogenates (especially the stimulation of glucose synthesis by AMP, and the evidence indicating that points of action of AMP are phosphofructokinase and fructose diphosphatase) support the concept that the reactions catalysed by phosphofructokinase and fructose diphosphatase are among those which limit the rate of glycolysis and gluconeogenesis respectively, and that the concentrations of intracellular modifiers, in particular AMP and ATP, regulate the activity of these enzymes. The fact that AMP at low concentrations (0·2mM) is a powerful inhibitor of fructose diphosphatase, as well as a powerful activator of phosphofructokinase, suggests that AMP is in the present system the most important single modifier among the various cell constituents which can influence the activities of the two enzymes, though ADP has similar effects on phosphofructokinase, the quantitative significance of which cannot yet be assessed. The concentration of AMP is a particularly apt 'signal' for regulating glycolysis and gluconeogenesis, as an energy-yielding and an energy-consuming process, because it is directly linked to the energy status of the cell, i.e. the concentration of ATP. The concentrations of AMP and ATP are governed by the equilibrium constant $K=[AMP][ATP]/[ADP]^2$ of the adenylate-kinase reaction. As the activity of this enzyme is sufficiently high to maintain the concentrations of the three reactants near equilibrium, a fall in the concentration of ATP will cause a rise in the concentration of AMP, and this, by activating phosphofructokinase, will increase the rate of ATP synthesis. On the other hand, if the adenine nucleotides of the cell are present predominantly as ATP, the AMP concentration will be low, phosphofructokinase blocked and the pathway of gluconeogenesis open.

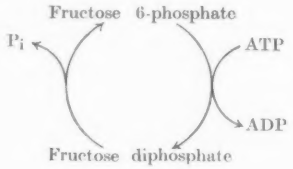
The kinetics of the AMP effect on phosphofructokinase are very intricate because they depend not only on the concentration of AMP but also on those of Mg^{2+} and of ATP (see Table 14) and, as the concentrations of cell constituents may differ from compartment to compartment within tissue homogenates, it is not possible to predict the degree of inhibition of activation from information on the overall concentration of AMP alone. The kinetics of the AMP inhibition of fructose diphosphatase appear to be less involved, though they depend on the concentration of Mg^{2+} (Table 14) as expected because of the formation of chelates between AMP and Mg^{2+}. Rather lower concentrations of AMP (below 0·1mM) are needed for an almost complete inhibition of fructose diphosphatase than for the activation of phosphofructokinase (above 0·1mM). These characteristics, according to available data, bear on the co-ordinated control of the two enzymes: at very low concentrations of AMP when fructose diphosphatase is active phosphofructokinase is inactive, being inhibited by ATP; as the concentration of AMP rises fructose diphosphatase becomes

Table 14. *Effects of various 'modifiers' on fructose diphosphatase and phosphofructokinase of liver*

The values illustrate that inhibitions depend on the presence of various substances in the medium. They are selected from data of Underwood & Newsholme (1965*a*,*b*), obtained with partially purified rat-liver enzymes. The substrate for fructose diphosphatase was fructose diphosphate and that for phosphofructokinase glucose 6-phosphate, phosphohexose isomerase being present. It is emphasized that the values indicate only the order of magnitude of the effects involved; there are complicating factors, such as the effects of enzyme dilution, that make precise statements on inhibitions difficult.

Metabolite	Concn. of substrate (mM)	Concn. of Mg^{2+} (mM)	Inhibition (%) Fructose diphosphatase	Inhibition (%) Phosphofructokinase
0·1mM-AMP	0·1	5	95	
0·05mM-AMP	0·1	0·5	98	
—	0·1	5	20	
—	0·25	0·5	15	
0·16mM-ATP	3·3	5		100
0·16mM-ATP, 0·3mM-AMP	3·3	5		18
1·2mM-ATP, 0·6mM-AMP	3·3	5		90
0·01mM-ATP	3·3	5		0
0·01mM-ATP, 0·5mM-citrate	3·3	5		50

inactivated before phosphofructokinase is activated. This avoids a wastage of energy through the occurrence of the futile cycle:



The data of Table 5 indicate that this cycle occurred in the homogenates (to which AMP was added) at a certain range of AMP concentrations, but this does not imply that it occurs *in vivo*.

It is not yet clear whether a physiological significance can be attributed to the inhibition of fructose diphosphatase by fructose diphosphate. So far inhibitory concentrations have not been demonstrated *in vivo*.

Intracellular distribution of enzyme inhibitors and activators, and the control of enzymes by active transport of modifiers. The overall concentration of ATP in homogenates was, in some experiments, high enough, considering the concentration of the de-inhibitors fructose 6-phosphate, fructose diphosphate, P_i and AMP, to cause a virtually complete inhibition of phosphofructokinase (Underwood & Newsholme, 1965*b*). In fact phosphofructokinase was not inactivated. This indicates that certain quantitative characteristics of the enzyme change on purification and dilution, as suggested by the observations of Underwood & Newsholme (1965*b*), or, more likely, that the concentration of ATP at the site of the enzyme was substantially below the average concentration in the homogenate. Two factors could be responsible for this. One is the uneven distribution of adenine nucleotides between the mitochondria and the cytoplasm or medium (see Frunder, Blume, Thielmann & Börnig, 1961; Loiselle & Denstedt, 1964), which causes the ATP concentration in the mitochondria to be substantially higher than in the soluble phase. The second factor is chelation of ATP with Mg^{2+} and Ca^{2+}.

It follows from these considerations that the movement of adenine nucleotides into and out of the mitochondria and other organelles could be a major factor in the regulation of metabolic processes. This is also true for other solutes that can be actively accumulated by mitochondria and that can affect the activity of pace-maker enzymes. Active transport into the mitochondria has been shown, for example, for Ca^{2+} (see Ernster & Lee, 1964; Drahota, Carafoli, Rossi, Gamble & Lehninger, 1965; Carafoli, Rossi & Lehninger, 1965; Chance, 1965), Mg^{2+} (Carafoli, Rossi & Lehninger, 1964), P_i (Greenawalt, Rossi & Lehninger, 1964; Rossi & Lehninger, 1964; Vasington & Greenawalt, 1964), citrate (Schneider, Striebich & Hogeboom, 1956; Gamble, 1965) and ATP and ADP (Carafoli *et al.* 1965). ATP, ADP, P_i and citrate, apart from being reactants in various processes, regulate, as mentioned above, the activity of phosphofructokinase; Ca^{2+} inhibits pyruvate kinase and pyruvate carboxylase, and Mg^{2+} activates these enzymes.

Though much attention has been paid to the mechanism of the active transport of these solutes into the mitochondria, the physiological significance of these energy-consuming movements has not yet been analysed. It may well be the regulation of enzyme activities. Movements of Ca^{2+} could contribute to the control of pyruvate kinase and pyruvate carboxylase. Under some physiological conditions these two enzymes, which control the fate of pyruvate (whether it forms acetyl-CoA or oxaloacetate), must not be fully active at the same time (see Krebs & Eggleston, 1965). The two enzymes are present in different cell compartments, pyruvate kinase being cytoplasmic and pyruvate carboxylase a mitochondrial enzyme. Both are inhibited by Ca^{2+}. Hence, if the concentration of Ca^{2+} is high in the cytoplasm, pyruvate kinase is inhibited and pyruvate carboxylase active, and the situation is reversed by transport of Ca^{2+} into the mitochondria.

Traffic in one direction of substances that cannot be metabolized, like Ca^{2+} or Mg^{2+}, would make no biological sense. Thus there must be mechanisms that bring about the release of these substances from the mitochondria into the cytoplasm. This might be entirely passive as Chance (1965) suggests, in which case it occurs continuously according to the concentration gradient and is counteracted by active transport in the opposite direction.

Origin of fructose diphosphate and triose phosphate in pigeon-liver homogenates. The assumption was made by Krebs (1964*b*) that fructose diphosphate and triose phosphates, which accumulate in the presence of AMP and the absence of lactate, are derived from gluconeogenic precursors. The present experiments indicate that they are predominantly formed from endogenous carbohydrate. However, this does not alter the conclusions previously drawn about the effects on fructose diphosphatase and phosphofructokinase and the mechanism by which lactate removes the accumulated intermediates.

Fructose diphosphatase as a rate-limiting step in gluconeogenesis. Exton & Park (1965) concluded from measurements of the concentrations of glucose and fructose 6-phosphates, fructose diphosphate and dihydroxyacetone phosphate that fructose diphosphatase is not a rate-limiting stage in gluconeogenesis from lactate in perfused livers from starved rats and that the formation of triose phos-

phate from pyruvate is the rate-limiting stage. The present experiments are in agreement with this concept, which is also supported by experiments with kidney cortex where substrates forming oxaloacetate without involvement of pyruvate carboxylase form glucose much more rapidly than lactate (Krebs, Bennett, de Gasquet, Gascoyne & Yoshida, 1963). Exton & Park (1965) thought that their conclusion was confirmed by the observation that fructose can give rise to glucose at a faster rate than lactate. This was based on the assumption that fructose would enter the gluconeogenic pathway solely at the stage of triose phosphate, an assumption that has recently been shown to be incorrect at the high fructose concentrations (20mM) used by Exton & Park (Di Pietro, 1964). The view that fructose diphosphatase can limit the rate of gluconeogenesis under certain conditions is not invalidated by the conclusion that it is not always a rate-limiting factor.

Role of phosphate acceptor in the control of glucose degradation in liver. It is well established that the concentration of ADP can regulate the rate of the aerobic degradation of substrates generally and this raises the question whether the stimulating effect of adenine nucleotides is, at least in part, due to the formation of ADP and its role as phosphate acceptor. It is possible that this was a component in stimulating the conversion of glucose into pyruvate in the pigeon-liver homogenates, but the following facts show that in this system the main stimulating effects of the adenine nucleotides were those on phosphofructokinase: (1) The stimulation of glucose degradation to pyruvate caused by AMP in the presence of adequate concentrations of NAD^+ was accompanied by a rise in the concentrations of fructose diphosphate and triose phosphates, and by a fall in those of 3-phosphoglycerate and hexose 6-phosphates (Tables 3, 4 and 11). (2) The increase in the rate of glucose removal when AMP was added was the same when sufficient NAD^+ was present to saturate the triose phosphate-dehydrogenase system and when the rate of this step was limited by lack of NAD^+ or by iodoacetate. In the first case, there were small increases in the concentrations of fructose diphosphate and triose phosphates; in the second, the large accumulation of these intermediates accounted quantitatively for the carbohydrate removed (see Tables 3, 4 and 12).

Wenner, Dunn & Weinhouse (1953) noted that the addition of phosphate acceptor did not affect the rate of formation of $^{14}CO_2$ from [^{14}C]glucose in rat-liver homogenates while NAD^+ (2mM), as in the present experiments, was an obligatory requirement for optimum rates of oxidation. Tiedemann & Born (1963), who studied the effects of adenine nucleotides on the glycolysis of homogenates from ascites-tumour cells, found that phosphate acceptors did not limit the rates and that adenine nucleotides had effects similar to those found with pigeon-liver homogenates. The similarity, of course, is restricted to the control of glucose degradation, as gluconeogenesis does not occur in ascites cells.

Note on the historical developments in the study of the regulatory properties of phosphofructokinase and fructose diphosphatase. A special role of phosphofructokinase in the regulation of glycolysis was first suggested by Cori (1942) on the basis of the observation that, under certain conditions, hexose monophosphate accumulated in muscle while the concentration of fructose diphosphate remained low and no lactate was formed. Cori (1942) concluded that 'this indicates that the reaction between fructose 6-phosphate and ATP . . . is a limiting factor as regards the rate at which lactic acid is formed and carbohydrate is oxidized'. Engelhardt & Sakov (1943) suggested that the activity of phosphofructokinase might be varied by reversible oxidations and reductions. The modern development of ideas about the mechanisms of modifying the activity of the enzyme began with the discovery by Lardy & Parks (1956) that the enzyme is inhibited by ATP, and their comment 'that this may help explain the lack of hexose diphosphate accumulation in stimulated muscle discussed by Cori'. The next stage was the discovery of Mansour & Mansour (1962) that the enzyme is activated by 3′,5′-(cyclic)-AMP, followed soon by the work of Passonneau & Lowry (1962*a*,*b*) showing that the inhibition by ATP is overcome by fructose 6-phosphate, fructose diphosphate and P_i, and that AMP and ADP activate the enzyme to some extent irrespective of the ATP concentration. Shortly afterwards Parmeggiani & Bowman (1963), Garland, Randle & Newsholme (1963) and Passonneau & Lowry (1963), almost simultaneously, reported the inhibition of phosphofructokinase by citrate.

The observations referred to were made on phosphofructokinase preparations obtained from a variety of different tissues. The enzyme from different sources of different origin seems to be susceptible to modifiers in the same way although there are quantitative differences. This applies to mammalian brain (Passonneau & Lowry, 1964), striated muscle (Passonneau & Lowry, 1962*a*,*b*), cardiac muscle (Mansour, 1963, 1965) and liver (Passonneau & Lowry, 1964; Underwood & Newsholme, 1965*b*), yeast (Ramaiah, Hathaway & Atkinson, 1964; Salas, Viñuela, Salas & Sols, 1965), liver fluke (Mansour & Mansour, 1962) and *Escherichia coli* (Atkinson & Walton, 1965).

The concept that fructose diphosphatase plays a role in the synthesis of glycogen from lactate seems to have been expressed first by Lardy (1949). It was referred to by Olson (1951) and by Pogell & McGilvery (1952) and elaborated by Krebs (1954). The inhibition of fructose diphosphatase by higher concentrations of fructose diphosphate was found independently by Weber (1964) and Krebs (1963, 1964*a*,*b*). The inhibition of fructose diphosphatase by AMP was simultaneously reported by J. S. Williams, D. E. Wilson & M. F. Utter (personal communication), Taketa & Pogell (1963) and Newsholme (1963).

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Comparative Detoxication

THE DETOXICATION OF AROMATIC ACIDS BY INVERTEBRATES: DETECTION OF AGMATINE CONJUGATES IN SCORPIONS

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1. Benzoic acid and *p*-nitrobenzoic acid were converted in the scorpion *Palamnaeus* into N^1-benzoylagmatine and N^1-*p*-nitrobenzoylagmatine. 2. Some benzoyl- and *p*-nitrobenzoyl-arginine were also formed and these compounds were probable precursors of the agmatine derivatives. 3. *p*-Nitrobenzoyl- and *p*-aminobenzoyl-arginine were formed in millipedes dosed with the aromatic acids. 4. Woodlice excreted *p*-amino- and *p*-nitro-benzoic acid as their glycine conjugates and no conjugation could be found in the crab *Gecarcinus* and a freshwater crayfish *Astacus*.

A wide variety of amino acids are used in the peptide detoxication of aromatic acids (Smith, 1964) and it is possible that the identification of a particular conjugation mechanism may be of some taxonomic value. Conjugations of aromatic acids with arginine have been shown to occur in a number of arachnids (Hitchcock & Smith, 1964) and may be characteristic of this Class, though a preliminary report (Smith, 1962) suggests that myriapods may also use this arginine detoxication.

Scorpions are usually classified as an arachnid Order, but when the Indian scorpion *Palamnaeus* sp. was examined by techniques used with spiders and ticks no arginine conjugates were found and the major metabolites of aromatic acids were unidentified basic compounds. We have now identified these scorpion metabolities as the agmatine derivatives of the administered aromatic acids. It is shown below that these are probably formed from arginine conjugates by a secondary decarboxylation reaction and that, as with other arachnids, scorpions form arginine conjugates as the primary detoxication products of aromatic acids.

EXPERIMENTAL

Reference compounds. All melting points are uncorrected. Agmatine sulphate (2g.) was dissolved in the minimum amount of water and stirred with 10g. of $NaHCO_3$ and 6g. of benzoyl chloride for 2hr. The mixture was acidified with 2N-HCl and the benzoic acid filtered off; the filtrate was evaporated to dryness *in vacuo* and extracted with 3 × 50ml. of acetone. Addition of ether to the acetone solution precipitated a small quantity of a salt of benzoylagmatine, which was recrystallized from methanol by the addition of ether as *N^1-benzoylagmatine carbonate*, m.p. 192° (decomp.) [Found: C, 56·4; H, 7·4; N, 20·9. $(C_{12}H_{18}N_4O)_2,H_2CO_3$ requires C, 56·6; H, 7·2; N, 21·1%]. The carbonate was very soluble in water and on the addition of aqueous picric acid formed a sparingly soluble *picrate*, m.p. 218° [Found: C, 46·6; H, 5·0; mol.wt. (Cunningham, Dawson & Spring, 1951), 450. $C_{18}H_{21}N_7O_8$ requires C, 46·7; H, 4·6%; mol.wt. 463].

Agmatine hydrochloride (2g.) was stirred in 20ml. of acetone–water (1:1, v/v) with 10ml. of N-KOH. *p*-Nitrobenzoyl chloride (1·9g. in 10ml. of acetone) was added and a further 10ml. of N-KOH added dropwise to maintain the pH at 7–8. The solution was filtered and the yellow solution concentrated *in vacuo* to remove acetone. The crystalline precipitate was removed and extracted with 10ml. of boiling water. Insoluble material was filtered off and the filtrate cooled to precipitate the impure *p*-nitrobenzoic acid salt of *p*-nitrobenzoylagmatine. The crude salt (300mg.) was acidified with N-HCl and extracted with ether to remove *p*-nitrobenzoic acid. The aqueous layer was evaporated to dryness and the residue crystallized from ethanol. The crude *p*-nitrobenzoylagmatine hydrochloride was converted into its *p*-nitrobenzoic acid salt, which was recrystallized from water. The *p-nitrobenzoylagmatine salt of p-nitrobenzoic acid* formed fine needles, m.p. 187–189° after losing water at 100° (Found: C, 45·9; H, 5·7. $C_{19}H_{22}N_6O_7,3H_2O$ requires C, 45·6; H, 5·6%). It was converted into the hydrochloride as described above and the *p-nitrobenzoylagmatine hydrochloride* had m.p. 188° (Found: C, 40·6; H, 6·4. $C_{12}H_{18}ClN_5O_3$ requires C, 41·0; H, 6·3%). It was very soluble in water and on the addition of picric acid formed the sparingly soluble *dipicrate*, m.p. 187–189° (Found: C, 38·6; H, 3·7; mol.wt. 381. $C_{24}H_{23}N_{11}O_{17},H_2O$ requires C, 38·2; H, 3·3%; mol.wt. 378). The *p*-nitrobenzoylagmatine was identical in its chromatographic and ionophoretic behaviour with the product present in solution obtained by incubating N^2-*p*-nitrobenzoyl-L-arginine over-

night with L-arginine decarboxylase (Sigma Chemical Co., St Louis, Mo., U.S.A.).

Other aroylamino acids were samples prepared previously (Hitchcock & Smith, 1964; Smith, 1958) or were commercial products.

Ring-labelled [^{14}C]benzoic acid was obtained from The Radiochemical Centre, Amersham, Bucks.

Animals. Scorpions were obtained from India (*Palamnaeus* sp., 10–20g.) or from France (*Euscorpius* sp., 0·5–1g.) and were kept in closed clear-plastic boxes with access to water. They were fed on mealworms or other small insects but survived readily without food for several months.

Millipedes were obtained from India (*Thyropygus* sp., 10–20g.) or France (a species of Schizothyllinae, 0·5–1g.) and kept in moist grass or moss without food.

Woodlice (*Ligia* sp., *Armadillidium* sp.) were collected on the sea shore and from gardens near London.

Land crabs (*Gecarcinus ruricola*, 150–250g.) were kept on damp moss without food. Freshwater crayfish (*Astacus fluviatilis*) were kept in a shallow layer of freshwater and fed on earthworms.

Ticks (*Boophilus decoloratus*) were obtained from the Cooper Technical Bureau, Berkhamstead, Herts.

Compounds were normally administered as their sodium salts in aqueous solution, at 0·2mg./g. by injection into the haemocoel, but woodlice were conveniently dosed by allowing them to feed on rice paper that had been soaked in an ether solution of the aromatic acid and dried.

Assay of nitrobenzoyl derivatives. This was carried out by $TiCl_3$ reduction and coupling with *p*-dimethylaminobenzaldehyde as described by Cohen & Smith (1964) for *p*-nitrobenzoic acid. Zones of chromatograms that contained nitro compounds were left to soak in the reagent mixture for 0·5hr. and colours measured as described previously.

Table 1. *R_F values of some aromatic acids and their amino acid conjugates*

Solvents were run on Whatman no. 1 or no. 4 paper until the front had moved about 15in. Solvents were: A, butan-2-one saturated with 2N-NH_3; B, ethanol–water (4:1, v/v); C, propan-1-ol–aq. NH_3 (sp.gr. 0·88) (7:3, v/v); D, butan-2-one–water–2N-acetic acid (200:100:1, by vol.); E, butan-1-ol–acetic acid–water (4:1:5, by vol.); F, butan-1-ol–aq. NH_3 (sp.gr. 0·88)–water (4:1:5, by vol.).

	R_F					
Solvent ...	A	B	C	D	E	F
Benzoic acid	0·1	0·8	0·9	0·1	0·8	0·4
N^2-Benzoylarginine	0·1	0·7	0·8	0·1	0·5	0·5
Benzoylglutamic acid	0	0·9	0·6	0·9	0·8	0·1
N^2-Benzoylglutamine	0·1	0·7	0·6	0·4	0·7	0·3
Benzoylglycine	0·1	0·8	0·7	0·9	0·9	0·6
N^2-Benzoylcitrulline	0·1	0·7	0·6	0·9	0·6	0·4
N^1-Benzoylagmatine	0·2	0·8	0·4	0·1	0·3	0·7
p-Nitrobenzoic acid	0·3	0·8	0·8	0·9	0·9	0·6
N^2-*p*-Nitrobenzoylarginine	0·1	0·6	0·7	0·4	0·5	0·5
N^2-*p*-Nitrobenzoylglutamine	0·1	0·6	0·6	0·5	0·3	0·3
p Nitrobenzoylglutamic acid	0	0·4	0·3	0·8	0·8	0·1
N^2-*p*-Nitrobenzoylornithine	0·5	0·8	0·9	0·9	0·9	0·8
p-Nitrobenzoylglycine	0·2	0·7	0·6	0·9	0·8	0·4
N^2-*p*-Nitrobenzoylcitrulline	0·3	0·8	0·8	0·9	0·9	0·7
N^1-*p*-Nitrobenzoylagmatine	0·9	0·9	0·9	—	0·8	0·7
Urea	0·2	0·7	0·6	0·4	0·6	0·4

Table 2. *Apparent migration to anode (+) or to cathode (−) of some conjugates of* p-*nitrobenzoic acid*

Distances were measured in cm. relative to *p*-nitrobenzoic acid. Ionophoresis was in Britton & Robinson buffer (British Drug Houses Ltd.) or 0·02N-NaOH or -HCl at 10v/cm. for 2hr.

	Migration (cm.)						
pH ...	12·7	10	8	6	4	3	1·3
p-Nitrobenzoic acid	0	0	0	0	0	0	0
N^2-*p*-Nitrobenzoylarginine	−9·0	−3·2	−2·8	−3·8	−6·0	−3·8	−1·5
N^2-*p*-Nitrobenzoylglutamic acid	+2·8	+0·5	+0·9	+1·0	−1·5	−1·0	0
N^2-*p*-Nitrobenzoylglutamine	−2·7	−1·2	−1·1	−1·5	−2·0	−1·0	0
N^2-*p*-Nitrobenzoylornithine	−5·2	−1·5	−1·7	−2·0	−3·5	−1·5	0
p-Nitrobenzoylglycine	−1·0	−0·5	−1·2	−0·4	−·05	−0·1	0
N^2-*p*-Nitrobenzoylcitrulline	0	0	0	0	−0·1	0	0
N^1-*p*-Nitrobenzoylagmatine	−15·2	−5·4	−4·5	−7·8	−8·4	−6·0	−2·3

Table 3. *Apparent migration to anode (+) or cathode (−) of conjugates of benzoic acid*

Distances were measured in cm. relative to benzoic acid. Ionophoresis was in Britton & Robinson buffer (British Drug Houses Ltd.) or 0·02 N-NaOH or -HCl at 10 v/cm.

	Migration (cm.)			
pH ...	1·3	4	6	12·7
Benzoic acid	0	0	0	0
N^2-Benzoylarginine	−5·6	−7·1	−8·9	−11·5
Benzoylglutamic acid	+20·0	+1·6	+2·1	+4·0
N^2-Benzoylglutamine	+1·2	−0·9	−3·0	+2·2
N^2-Benzoylcitrulline	0	−1·1	−0·6	+1·4
N^2-Benzoylornithine	−4·3	0	−8·4	+2·0
N^1-Benzoylagmatine	−8·3	−14·7	−11·4	−13·7
Duration of ionophoresis (hr.)	3·5	2·5	4·0	2·5

Table 4. *Conjugation of* [^{14}C]*benzoic acid in* Palamnaeus

Duration of experiment (days)	Extracted radioactivity (μc)	Percentage of dose recovered				
		Unchanged	N^2-Benzoyl-glutamic acid	N^2-Benzoyl-glutamine	N^2-Benzoyl-arginine	N^2-Benzoyl-agmatine
2	0·65	69*	—	—	0·87*	—
2	0·63	58*	2·8*	0·0*	5·7*	—
2	0·89	44*	0·93*	0·0*	0·68*	—
2	0·91	71	0·0*	—	0·71*	29
2	0·86	62	0·74*	—	0·30*	38

* Assay by dilution analysis; other assays were by paper-strip counting.

Recovery of 10–100 μg. of *p*-nitrobenzoic acid, after paper chromatography, was quantitative.

Tissue homogenates containing nitro compounds were mixed with 5 vol. of acetone and centrifuged at 1000*g*. The supernatant was concentrated in a rotary evaporator and transferred quantitatively to a 7·5 cm.-wide strip of Whatman no. 1 paper, which was developed in solvent F. Zones containing nitro compounds were eluted and rechromatographed in solvent E before assaying as described above.

Extraction of metabolites. Animals were immobilized, if necessary, by freezing to −20° and the integuments removed. The soft internal parts were scraped out and ground with acetone–water (4:1, v/v) in a Potter–Elvehjem homogenizer. After centrifugation at 1000*g*, the supernatant was concentrated to small bulk for chromatographic or ionophoretic experiments.

In [^{14}C]benzoic acid experiments with scorpions integument and legs were cut in small pieces and added to the acetone homogenate of internal organs (100 ml./20 g. of scorpion), which was refluxed on a water bath for 1 hr. The insoluble part was centrifuged off and refluxed for a further 1 hr. with 15 ml. of water. Most of the radioactivity injected was found in the acetone extract and the rest in the aqueous extract.

Radioactive assays and scanning. These were carried out as described previously (Gessner & Smith, 1960; Hitchcock & Smith, 1964).

Paper chromatography and ionophoresis. These were carried out under conditions used previously (Hitchcock & Smith, 1964; Smith, 1958), with the same methods for detection of spots. Agmatine derivatives gave no ninhydrin reaction but with the Sakaguchi reagents gave a colour similar to that of the arginine conjugates. The degree of separation between conjugates is shown in Tables 1, 2 and 3.

RESULTS

Metabolism of [^{14}C]*benzoic acid.* Scorpions (*Palamnaeus* sp.) were injected with 2 mg. of [^{14}C]benzoic acid and kept for 2 days in closed plastic boxes. They were extracted as described above and the box was rinsed with water, which was added to the extract. The extract was divided into portions and analysed by dilution analysis for benzoic acid, benzoylglutamic acid, benzoylglutamine and benzoylarginine as described by Hitchcock & Smith (1964).

Other portions of the extract containing about 0·01 μc of radioactivity were applied to chromatograms or ionophoretic strips with 100 μg. of the reference *N*-benzoylamino acids. In quantitative experiments, strips were scanned for radioactivity after ionophoretic separation at pH 6.

Trace amounts of benzoylglutamic acid and benzoylarginine were detected (Table 4) in addition to the major metabolite, benzoylagmatine. Other conjugates of benzoic acid were not observed.

Metabolism of p-*nitrobenzoic acid and* p-*aminobenzoic acid.* Scorpions or millipedes were dosed, left for 48hr. at room temperature and extracted as described above. Initial large-scale chromatograms, as described by Hitchcock & Smith (1964), were run in solvent F and bands, after location, were eluted in aq. 0·1N-ammonia for further characterization.

Millipedes (*Thyropygus* and Schizothyllinae) normally yielded an extract giving two zones at R_F 0·5 and 0·6. The zone at R_F 0·6 was identified as unchanged *p*-nitrobenzoic acid, and the zone at R_F 0·5 gave the colour reactions of *p*-nitrobenzoylarginine and was chromatographically and ionophoretically identical with this compound in each of the solvent systems or buffers of Tables 1, 2 and 3. In some experiments traces of *p*-nitrobenzoylglutamic acid were present in *Thyropygus* extracts. When *p*-aminobenzoic acid was injected into *Thyropygus* and the animals were extracted a week later, only *p*-aminobenzoylarginine was detected by its chromatographic and ionophoretic properties.

The scorpion *Euscorpius* yielded two bands in solvent system F, one of which consisted of unchanged *p*-nitrobenzoic acid, at R_F 0·6, and the second, at R_F 0·1, gave the colour reactions of *p*-nitrobenzoylglutamic acid and behaved identically in each of the solvent systems and buffers of Tables 1 and 2.

The scorpion *Palamnaeus*, in most experiments, yielded two bands in solvent F, one of which, at R_F 0·6, was unchanged *p*-nitrobenzoic acid, and the second, at R_F 0·7, gave a positive Sakaguchi reaction. After elution it behaved identically with *p*-nitrobenzoylagmatine in each of the systems and buffers of Tables 1 and 2. Some *Palamnaeus* specimens gave extracts that contained very weak zones in solvent system F with R_F 0·1, and large amounts of this compound were found in extracts of a scorpion that died during the experiment. This zone was identified chromatographically and ionophoretically as *p*-nitrobenzoylglutamic acid.

Scorpions (*Palamnaeus* sp.) were also dosed with *p*-nitrobenzoic acid after preliminary treatment with neomycin. This was injected daily for 3 days (10μg./scorpion) or provided as a 1% solution in water during the week before injection of *p*-nitrobenzoic acid. No significant change was seen in the nature of the metabolites.

The terrestrial crustaceans *Ligia* sp. and *Armadillidium* sp. were dosed with *p*-aminobenzoic acid or *p*-nitrobenzoic acid as described above and the excreta were ground with a few drops of water and chromatographed in solvent F. Only one zone was found that differed from that of the aromatic acid administered.

These bands were eluted with 0·1N-ammonia and the eluate was examined in each of the systems of Tables 1 and 2. In each system the eluate behaved identically with the glycine conjugate of the aromatic acid used.

In experiments with the land crab *Gecarcinus* and the freshwater crayfish *Astacus*, no evidence of amino acid conjugations could be found.

Metabolism of some conjugates in arachnids. Twelve ticks were injected with 20μg. each of the conjugates of *p*-amino- or *p*-nitro-benzoic acid dissolved in 2μl. of water. They were kept at 32° for 2 days and extracted as described by Hitchcock & Smith (1964), and the extracts were examined ionophoretically and chromatographically for metabolites. Small amounts of unchanged conjugates were detected and some hydrolysis to the aromatic acids occurred, but the conjugates of *p*-nitrobenzoic acid with L- and DL-arginine, L-citrulline, L-ornithine and L-glutamine were all converted into *p*-nitrobenzoylglutamic acid. No other conjugates were detected. Similar results were obtained when the *p*-aminobenzoyl conjugates were administered, *p*-aminobenzoylglutamic acid being formed in each case.

When scorpions (*Palamnaeus* sp.) were dosed with 1mg. of *p*-nitrobenzoyl-L-arginine in 0·2ml. of water and extracted after 2 days, *p*-nitrobenzoylagmatine was found along with traces of *p*-nitrobenzoic acid and unchanged *p*-nitrobenzoylarginine.

Metabolism of conjugates in tissue homogenates. Mixed tissues from the scorpion body were ground with aq. 0·8% sodium chloride in a Potter–Elvehjem homogenizer to give 3ml. of homogenate/scorpion. The homogenate (1ml.) was incubated for 2hr. at 37° with 0·5mg. of the N^2-*p*-nitrobenzoyl derivative of L-arginine, L-ornithine or L-citrulline and 5ml. of buffer solutions ranging from pH 3 to 10. McIlvaine's (1921) citrate–phosphate buffer (0·1M) was used between pH 3 and 8 and Sörensen's (1912) glycine buffer (0·1M) between pH 8·5 and 10.

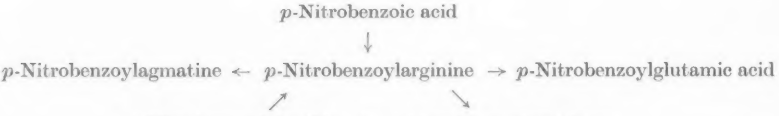
The solutions were deproteinized by the addition of 30ml. of acetone, concentrated to small bulk and examined in each of the chromatographic systems of Table 1. Different products were found at different pH values (see Table 5), and no reaction occurred in the control tubes containing boiled homogenate.

In similar quantitative experiments with *p*-nitrobenzoyl-L-arginine as substrate rates of formation of metabolites (in μg./scorpion/hr.) were 50, 81, 82 and 68 for *p*-nitrobenzoylagmatine, 112, 150 and 75 for *p*-nitrobenzoylglutamic acid, and 92, 95 and 100 for *p*-nitrobenzoylornithine.

Table 5. *Formation of metabolites from some conjugates of* p*-nitrobenzoic acid*

Homogenate from scorpions was incubated at 37° in buffer with 0·5 mg. of substrate.

Substrate	pH range for optimum formation				
	p-Nitrobenzoyl-agmatine	*p*-Nitrobenzoyl-ornithine	*p*-Nitrobenzoyl-citrulline	*p*-Nitrobenzoyl-glutamic acid	*p*-Nitrobenzoyl-arginine
p-Nitrobenzoylarginine	4·5–6·0	8·0–10·0	Not found	6·5–8·5	—
p-Nitrobenzoylornithine	Not found	—	5·5–6·5	Not found	Not found
p-Nitrobenzoylcitrulline	Not found	4·5–6·5	—	7·0 (trace)	5·5–8·5



Scheme 1.

Values for the rate of conversion of *p*-nitrobenzoyl-L-ornithine into *p*-nitrobenzoylcitrulline were 95 and 70 μg./scorpion/hr.; *p*-nitrobenzoyl-L-citrulline was converted into the ornithine derivative at 70 μg./scorpion/hr. and into *p*-nitrobenzoylarginine at a maximum rate of 30 μg./scorpion/hr.

Assays of the arginase activity in the homogenates from six scorpions by using the urease micro-diffusion technique (Glick, 1963) were in the range 0·7–3·1 μmoles of urea/min. at 25°.

DISCUSSION

The small amount of arginine conjugation detected in *Palamnaeus* dosed with aromatic acids and the demonstration that injected *p*-nitrobenzoylarginine was rapidly decarboxylated *in vitro* by scorpion homogenates supports the view that scorpions use arginine as the amino acid in the peptide detoxication as do the other arachnids. These observations reinforce the suggestion made by Hitchcock & Smith (1964) that the arginine conjugation is a biochemical characteristic of the arachnid Class.

Although all the arachnids so far studied have formed arginine conjugates a notable feature of the metabolism of aromatic acids in this group has been the multiplicity of other conjugated forms of aromatic acids found. Thus *Palamnaeus* extracts always contained small amounts of glutamic acid conjugates and these were major metabolites in *Euscorpius*. Extracts of ticks had both arginine and glutamic acid conjugates, and extracts of spiders and harvestmen usually contained glutamine and glutamic acid conjugates as well as the primary arginine conjugates. It seems probable that all of these may have been derived from a primary detoxication product, which in each case was the arginine conjugate of the aromatic acid administered (see Scheme 1), and it is these rather than the glutamic acid conjugates that merit the trivial name of arachnuric acids.

The mechanism by which the arginine conjugates were converted into the secondary metabolites is not clear. In experiments with homogenates the major product of enzyme action on *p*-nitrobenzoyl-arginine depended on the pH of the reaction mixture. The major metabolite *in vivo*, *p*-nitrobenzoyl-agmatine, was formed at about pH 5 *in vitro* but at pH 9 the major product was *p*-nitrobenzoyl-ornithine, and at pH 7 was *p*-nitrobenzoylglutamic acid. The decarboxylation of *p*-nitrobenzoyl-arginine in homogenates or intact scorpions occurred equally well in the presence of neomycin and, though bacterial arginine decarboxylases have pH optima near 5 (Gale, 1946), it seems unlikely that the formation of agmatine conjugates is due to bacterial contamination.

Palamnaeus homogenates also catalysed reactions analogous to those of the normal urea cycle and contained an active true arginase, but *in vivo* neither *p*-nitrobenzoyl-ornithine nor -citrulline could be identified as an intermediate in the metabolism of *p*-nitrobenzoic acid or *p*-nitrobenzoylarginine. The *p*-nitrobenzoylglutamic acid, which was formed by all arachnids to some extent *in vivo*, was only formed from *p*-nitrobenzoyl-arginine in homogenates.

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Studies of the Proteins, Peptides and Free Amino Acids of Mature Bovine Enamel

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1. The organic matrix of enamel from erupted bovine teeth has been found to be composed mostly of small peptides containing principally aspartic acid, glycine, glutamic acid and serine. 2. A small amount of higher-molecular-weight components has been isolated by various procedures. One non-diffusible fraction was found to be heterogeneous in the ultracentrifuge, and composed principally of material that by gel filtration indicated a molecular weight greater than 30 000. These components were largely carbohydrate in nature (glycoproteins and glycopeptides), containing only small amounts of amino acids.

Previous studies have described several methods for the isolation of the soluble and insoluble components of the organic matrix of mature bovine enamel essentially free from contamination with either dentinal or cemental collagen (Glimcher, Friberg & Levine, 1964*c*). The amino acid composition of the whole organic matrix, as well as of a number of soluble and insoluble fractions, differed markedly from that of both collagen and proteins of embryonic enamel (Glimcher *et al.* 1964*c*; Eastoe, 1960, 1963; Piez, 1961; Glimcher, Mechanic, Bonar & Daniel, 1961; Glimcher, Mechanic & Friberg, 1964*b*). Further, in contrast with the proteins of embryonic bovine enamel, most of the amino acid-containing constituents were readily diffusible through dialysis sacs or retarded on columns of Sephadex G-25 resin (Glimcher *et al.* 1964*c*). These findings, coupled with the fact that over 90% of the enamel proteins was lost during the development of the tooth, suggested that maturation of the enamel was accompanied by degradation of the matrix proteins, especially those components rich in proline and histidine but poor in serine (Glimcher *et al.* 1964*b*,*c*). The present paper deals with the further characterization of the proteins, peptides and free amino acid constituents of the organic matrix of enamel from erupted mature bovine teeth.

EXPERIMENTAL

Tissue source. The four central permanent erupted incisor teeth of 24–40-month-old steers were used. After dissection from the lower jaw, the crown of the tooth was separated from the root by sectioning the tooth approx. 3–5mm. above the gingival margin, and the pulp chambers were cleaned. Very heavily stained, cracked or pitted teeth were discarded.

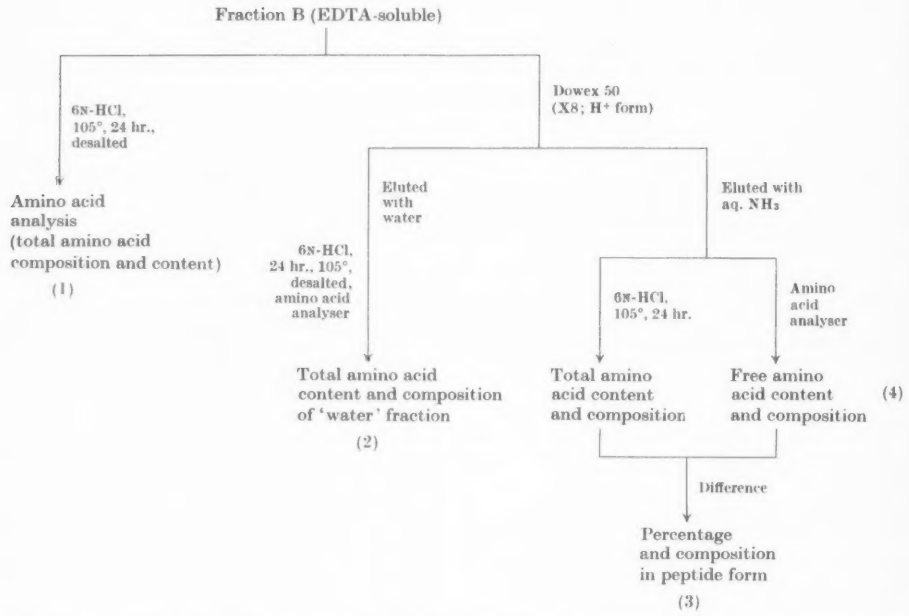
Isolation of the organic constituents of the enamel from selected areas of the labial surface of the tooth crown. The tooth crowns were placed in 0·5M-EDTA, pH 8·3, at 2° or 25° for 48hr. with toluene added for bacteriostasis. At the end of this period, the coronal cementum (Glimcher, Friberg & Levine, 1964*d*) was dissected free from the underlying enamel, and the tooth surface brushed gently. The tooth crowns were again placed in 0·5M-EDTA, pH 8·3, for approx. 4hr., their surfaces brushed briskly in EDTA, and rinsed in double-distilled demineralized water. After they were dried at 25° over P_2O_5 in vacuum desiccators, the pulp chambers were filled with dental acrylic resin and all but a 1·0cm. × 1·0cm. area of the central portion of the labial surface of the enamel was covered with (finger)nail varnish (Cutex; Chesebrough–Ponds Inc., New York, N.Y., U.S.A.) (Glimcher *et al.* 1964*c*).

Two methods were used to dissolve the mineral phase (and most of the organic matrix) of the selected area of crown enamel. In the first method, the tooth crowns, prepared as described, were placed in covered polystyrene trays containing 0·5M-EDTA, pH 8·0, at 8–10°. The EDTA solution was circulated continuously by means of a peristaltic pump and changed at 48hr. intervals. The teeth were carefully observed during the procedure, and coronal and cross-sections made of randomly selected teeth so that the procedure could be terminated while an adequate thickness of undecalcified enamel still remained covering the dentine over the exposed labial surface. All the EDTA solutions were pooled and analysed as a single sample. About 5000 teeth were used for this experiment. In the second procedure, the tooth crowns were placed in the polystyrene trays and covered with double-distilled demineralized water. Then HCl gas was bubbled in at a constant rate to maintain the pH at 2·3–2·8 and the solution circulated by means of a peristaltic pump. About 2000 teeth were used in this experiment.

A third method was used to obtain an ethanol-insoluble

Selected area of enamel of tooth crowns
Exposed to circulating EDTA, pH 8·0, 10°
EDTA Insoluble
Acidified to pH 1·5, 2°
EDTA-insoluble, acid-insoluble (1)
EDTA-insoluble, acid-soluble (2)
EDTA-soluble
Dialysed against water, pH 7·0
Non-diffusible, water-soluble
Sephadex G-25
Fraction A Mol. wt. > 3500 (12)
Fraction B Mol. wt. < 3500 (13)
Acidified to pH 1·5
Half dialysed against neutralized water
EDTA precipitate
Half filtered through Sephadex G-25, pH 2·5, 5% acetic acid
Non-diffusible, water-insoluble (7)
Non-diffusible, water-soluble (8)
6 M-Urea
Insoluble precipitate
Soluble (9)
Filtered through polyacrylamide P-30 column in 6 M-urea
Mol. wt. > 30000 (10)
Mol. wt. < 30000 (11)
Fraction A (3)
Fraction B (4)
Fraction C (5)
Fraction D (6)
Mol. wt. > 3500
Mol. wt. < 3500

Scheme 1. Procedures employed for the isolation and separation of the EDTA-soluble, EDTA-insoluble, diffusible and non-diffusible proteins and peptides of erupted bovine enamel. The numbers in parentheses refer to the columns in Table 1.


Fraction B (EDTA-soluble)
6N-HCl, 105°, 24 hr., desalted
Dowex 50 (X8; H+ form)
Amino acid analysis (total amino acid composition and content)
(1)
Eluted with water
Eluted with aq. NH3
6N-HCl, 24 hr., 105°, desalted, amino acid analyser
6N-HCl, 105°, 24 hr.
Amino acid analyser
Total amino acid content and composition of 'water' fraction
(2)
Total amino acid content and composition
Free amino acid content and composition
(4)
Difference
Percentage and composition in peptide form
(3)

Scheme 2. Procedures employed in the chromatographic separation of the peptides and free amino acids of EDTA-soluble fraction B. The numbers in parentheses refer to the columns in Table 2.

fraction of the organic matrix. In this technique, whole tooth crowns were placed in EDTA and the coronal cementum was removed as described. The crowns were then placed in fresh 0·5 M-EDTA, pH 8·3, at 2° for 4–6 hr., during which time the surfaces were brushed briskly several times. The tooth crowns were removed from the EDTA solution, washed thoroughly with water and dried. The labial surface of the tooth crowns was then stained with haematoxylin, which was found to be a very sensitive index of the presence of small remnants of the cementum. If there was positive-staining material present the crowns were placed for various times in ethanol to which was added conc. HCl to give a final concn. of 0·1 N, and the softened surface layer was scraped gently with a sharp scalpel blade. The procedure was repeated until the surface of the enamel did not stain with haematoxylin. The crowns were then cut into 1000 μ-thick cross-sections on a modified Gillings–Hameo apparatus (Hameo Machine Co. Inc., Rochester, N.Y., U.S.A.) and slabs of enamel dissected free from the dentine by an air-abrasive technique (Friberg, Levine & Glimcher, 1964). The surface of the enamel slabs closest to the dentino-enamel junction were then further abraded by using dental brushes.

The enamel slabs were placed in ethanol made 0·1 N with respect to HCl at room temperature and the solution was stirred gently. The decalcified precipitate was separated by centrifugation, washed with ethanol made 0·1 N with respect to HCl and dried. Except for amino acid analyses, this material was not further utilized in this study.

Recovery and fractionation of the EDTA- and hydrochloric acid-soluble proteins, peptides and amino acids. The procedures followed are outlined in Schemes 1, 2 and 3. The EDTA solutions were filtered or centrifuged to obtain the EDTA-insoluble residue and a clear supernatant. The EDTA-insoluble residue, representing only a very small portion of the organic matrix, was washed quickly with water, and then placed in water adjusted to pH 2·0 with HCl at 2° and stirred. After 48 hr. the insoluble residue (EDTA-insoluble acid-insoluble fraction) was separated from the supernatant (EDTA-insoluble acid-soluble fraction). Both fractions were used only for amino acid analysis.

Most of the EDTA was precipitated from the original clear supernatant (EDTA-soluble fraction; Scheme 1) by adjusting the pH to 1·5 at 2°. The clear supernatant was concentrated on a rotary evaporator, and the procedure repeated several times (Glimcher *et al.* 1964*c*). The final supernatant was adjusted to pH 2·0–2·5 and divided equally into two portions. One half of the solution was dialysed exhaustively against water adjusted to approx. pH 7·0 with aq. NH_3. The soluble and insoluble fractions of this non-diffusible fraction were separated by centrifugation. The insoluble residue was used only for amino acid analysis. The water-soluble fraction was freeze-dried and a portion used for chemical analyses. The remainder of this fraction was dissolved (with difficulty) in 6 M-urea in 0·15 M-tris–HCl buffer, pH 7·4. A portion of the material soluble in 6 M-urea was used for analytical ultracentrifugation studies, and the

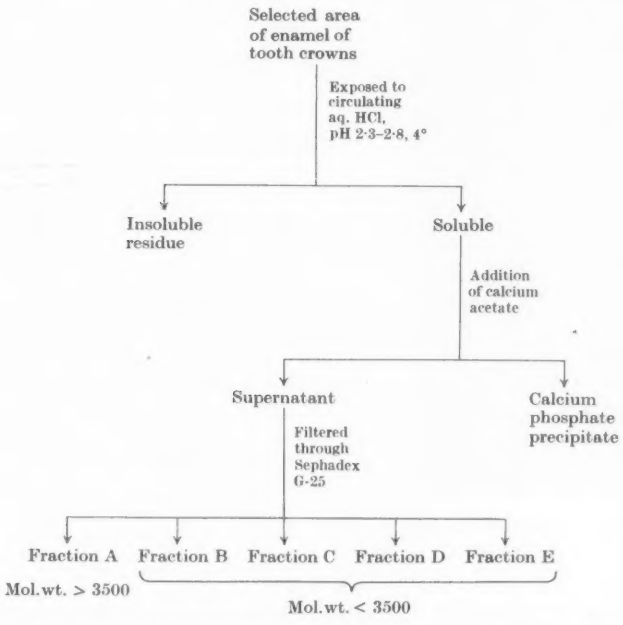
remainder of the soluble portion filtered through a column (0·9 cm. × 48 cm.) of Bio-Gel P–30 resin (50–150 mesh) (copolymer of acrylamide and methylenebisacrylamide; Bio-Rad Laboratories, Richmond, Calif., U.S.A.) equilibrated in 6 M-urea. Two fractions were collected: one that was excluded from the column and therefore represented material of mol.wt. greater than 30000, and a second that was retarded on the column and therefore contained material of mol.wt. less than 30000. The urea was removed by trituration with ethanol and the material was dried. Because of the small amount of material in these fractions and its relative insolubility, no further physicochemical studies were undertaken.

The other half of the EDTA-soluble fraction was filtered through a column (3 cm. × 170 cm.) of Sephadex G-25 resin equilibrated with 5% acetic acid, pH 2·5, at 2°, and four fractions were collected. Bovine serum albumin, NaCl and the peptides glycyl-L-aspartic acid, glycyl-L-leucylglycine, tetraglycine and pentaglycine, as well as the amino acids glycine and aspartic acid, were used to standardize the column run under identical conditions.

Fraction B (see Scheme 1) was further subjected to the procedures outlined in Scheme 2. A portion was weighed out and hydrolysed in triple-distilled constant-boiling 6 N-HCl for 24 hr. at 105°. The hydrolysate was evaporated to dryness three times and desalted on a column of Dowex 50 (X2; H^+ form) resin, and subjected to amino acid analysis. This represented the total amino acid composition and content of fraction B. The remainder of the material was weighed, dissolved in 0·01 N-HCl and chromatographed on a column (2·4 cm. × 45 cm.) of Dowex 50 (X8; H^+ form) resin. The sample was eluted with water and the first 4–6 column volumes eluted with water were separated from the material eluted thereafter with aq. N-NH_3. The percentage and composition of the material in the water and aq.-NH_3 fractions existing in peptide form and as free amino acids were determined by chromatographing unhydrolysed and hydrolysed portions on the automatic amino acid analyser.

The HCl-soluble material was separated as shown in Scheme 3. No HCl-insoluble precipitate was recovered. In this case most of the calcium and phosphate was removed before filtration through Sephadex G-25 by the addition of calcium acetate. Fractions C and D of the HCl-soluble material obtained by Sephadex G-25 filtration were further fractionated in the manner described for fraction B (Scheme 2). To assess the possible hydrolysis of peptides on the Dowex 50 (X8; H^+ form) resin, known amounts of several peptides (e.g. glycyl-L-aspartic acid and glycyl-L-leucylglycine) were chromatographed on the same column under conditions identical with those used for the enamel peptides. The material eluted from the columns was then analysed directly in the automatic amino acid analyser without further hydrolysis to determine the amounts, if any, of free amino acids liberated.

To minimize the effect of peptide-bond hydrolysis that might have occurred during the repeated precipitations of



Scheme 3. Procedures employed for the isolation and separation of the HCl-soluble proteins and peptides of erupted bovine enamel.

Table 1. *Amino acid compositions of the EDTA-soluble and -insoluble components of the organic matrix of erupted bovine dental enamel*

The numbers at the top of the columns refer to the fractions shown in Scheme 1. Values are expressed as residues/1000 total amino acid residues.

	EDTA-insoluble		EDTA-soluble: Filtered through Sephadex G-25 resin				EDTA-soluble: Non-diffusible							
									Soluble in 6 M-urea			Water-soluble§ Filtered through Sephadex G-25 resin		
	Acid-insoluble (1)	Acid-soluble (2)	Fraction A (3)	Fraction B (4)	Fraction C (5)	Fraction D (6)	Water-insoluble (7)	Water-soluble (8)	Total (9)	Mol.wt. > 30000 (10)	Mol.wt. < 30000 (11)	Fraction A (12)	Fraction B (13)	Ethanol-insoluble
Cystine (half)*	3	—	—	< 1	—	—	—	—	7	8	—	—	5	10
3-Hydroxyproline	—	—	—	—	—	—	—	—	—	—	—	—	—	—
4-Hydroxyproline	58	—	—	—	—	—	—	—	—	—	—	—	—	5
Aspartic acid	37	343	153	85	70	44	266	223	140	148	99	82	66	215
Threonine	11	19	90	31	15	12	26	37	36	36	35	54	43	35
Serine	20	345	127	64	51	57	186	167	130	126	148	199	258	201
Glutamic acid	58	30	150	129	134	71	100	141	124	129	99	197	138	77
Proline	214	21	78	28	8	25	33	42	44	52	Trace	56	28	42
Glycine	248	142	161	511	573	459	153	123	190	189	197	152	227	115
Alanine	82	21	‡	58	31	36	51	42	67	68	63	92	82	73
Valine	46	10	60	27	14	17	21	58	39	38	42	40	26	36
Methionine†	< 1	2	1	10	9	4	4	30	—	—	—	2	—	7
Isoleucine	32	‡	22	‡	‡	16	13	28	29	29	28	23	15	20
Leucine	74	‡	41	34	‡	20	27	35	38	38	35	39	22	43
Tyrosine	11	Trace	2	4	82	Trace	Trace	Trace	3	Trace	21	6	10	5
Phenylalanine	28	5	14	8	Trace	179	15	14	23	20	35	15	24	22
Hydroxylysine	2	—	—	—	—	Trace	—	—	—	—	—	—	—	2
Lysine	57	45	54	8	8	23	54	30	58	57	63	17	24	53
Histidine	12	8	19	Trace	Trace	10	20	13	23	22	28	14	24	11
Arginine	7	11	29	3	5	27	31	16	50	40	106	12	9	30

* Recovered as cysteic acid.

† Recovered as methionine and methionine sulphoxide.

‡ In some cases in which no values are given, the chromatographic peak in the region that ordinarily corresponded to these amino acids was broadened and asymmetric and had a different E_{570}/E_{440} ratio. Further hydrolysis of these samples at 115° for 24 hr. in 6 N-HCl did not change these properties.

§ Not acidified before dialysis (separate experiment: see the text).

the EDTA at acid pH, another experiment was carried out in which the EDTA solution removed from the polystyrene boxes was alternately dialysed against neutral water and concentrated until it was free of EDTA, equilibrated for several hours at pH4·4 at 2° and filtered through a column (3·0cm.×250cm.) of Sephadex G-25 equilibrated in pyridine–acetic acid buffer, pH4·4 (Scheme 1).

Analytical procedures. Amino acid analyses were performed on samples hydrolysed in triple-distilled constant-boiling 6N-HCl for 24hr. at 105°. A commercial model (Phoenix Precision Instrument Co., Philadelphia, Pa., U.S.A.) of an automatic amino acid analyser described by Piez & Morris (1960) was used for the amino acid analyses.

The content of hexoses was determined by the anthrone method (Scott & Melvin, 1953), with glucose as a standard.

Ultracentrifugation. Sedimentation coefficients were measured at pH7·4 in 6M-urea in 0·15M-KCl at 20° in a double-sector cell by using a synthetic boundary in a Spinco model E analytical ultracentrifuge, and the values were corrected to 20° in water ($S_{20,w}$).

Thin-layer chromatography. Weighed portions of material were hydrolysed in 0·1N-HCl for 24hr. at 100°. A sample was used for the identification of hexoses by thin-layer chromatography on cellulose powder with ethyl acetate–pyridine–water (12:5:4, by vol.) as the solvent (Randerath, 1963). Another sample was further hydrolysed in 4N-HCl at 105° for 5hr. and used to identify the hexosamines by thin-layer chromatography as described. The number of μmoles of amino acid/μmole of hexose in the samples was determined by amino acid analyses and total anthrone determinations of measured volumes of the samples.

RESULTS

EDTA preparations. Table 1 summarizes the amino acid compositions of the fractions outlined in Scheme 1. Fig. 1 shows a typical elution curve of the EDTA-soluble fraction filtered through Sephadex G-25 resin. The fractions eluted in the region corresponding to proteins or peptides of mol.wt. greater than 3500 and excluded from the column were barely detectable when analysed undiluted at 276mμ. When the material in this region was pooled and dried, it constituted less than 10% of the total sample by weight. Most of the EDTA-soluble material was eluted in the region of small peptides and amino acids. Except for the acid- and EDTA-insoluble fraction, neither 3- nor 4-hydroxyproline was noted in the chromatograms, although the presence of very small amounts would

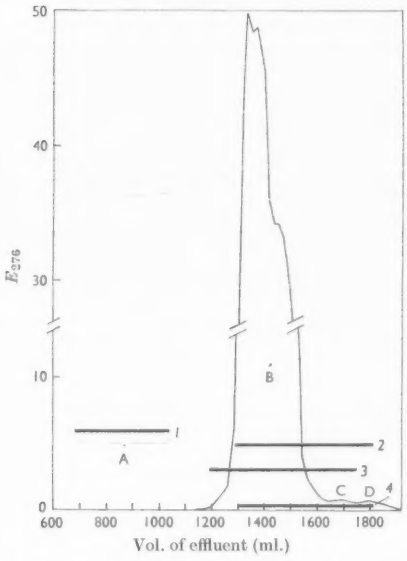


Fig. 1. Typical elution curve of the EDTA-soluble peptides of erupted bovine enamel filtered through a column (3cm.×170cm.) of Sephadex G-25 resin in 5% acetic acid, pH2·5, at 2°. The horizontal bars correspond to the positions of: *1*, bovine plasma albumin; *2*, amino acids (glycine and aspartic acid); *3*, peptides (mol.wt. 250–300); *4*, salt.

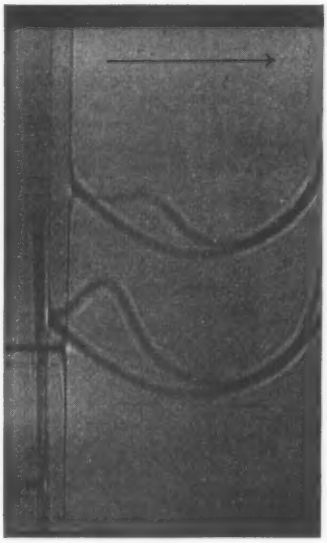


Fig. 2. Sedimentation-velocity pattern obtained from the non-diffusible water-soluble fraction of the EDTA-soluble portion of adult enamel matrix run in 6M-urea in 0·15M-KCl, pH7·4, at 20°. The top cell represents a solution at a concentration of approx. 0·4% (w/v) and the bottom cell approx. 0·8% (w/v). The time was 80min. after reaching the maximum speed of 50780 rev/min. The phase angle is 55°.

not have been detected owing to the low molar extinction coefficient of the hydroxyprolines after reaction with ninhydrin. The relatively high concentration of 4-hydroxyproline and glycine makes it likely that the acid- and EDTA-insoluble residue contained a considerable proportion of collagen. The very high proline content, however, suggests the presence of components similar to those isolated from embryonic bovine enamel (Glimcher *et al.* 1964*b*).

Because of the extremely high content of phenylalanine in fraction D (EDTA-soluble), and because substances of an aromatic nature may be strongly adsorbed on dextran gels, the tripeptide glycyl-L-phenylalanyl-L-phenylalanine (mol.wt. 369·4) was chromatographed on the Sephadex G-25 resin column. It was greatly retarded, and was eluted in the general region corresponding roughly to that of fraction D.

The major portion of the non-diffusible material that was obtained by dialysis against water, and that was acidified only to pH 4·7 just before Sephadex G-25 filtration, was excluded from the column and was therefore of mol.wt. greater than 3500. One other fraction of lower molecular weight was also collected. The amino acid compositions of these two fractions are also given in Table 1.

The non-diffusible water-soluble fraction (obtained after acidification) was extremely difficult to redissolve after freeze-drying, even when warmed to 35–40° in 6M-urea. Ultracentrifugation (Fig. 2) of that fraction soluble in 6M-urea showed that most of the material was represented by a boundary with a sedimentation coefficient ($S_{20,w}$) of approx. 1·5S. There is a pronounced leading edge to this major boundary and asymmetry of the peak indicating heterogeneity. Two fractions were separated from this material by gel filtration on

Table 2. *Amino acid compositions of the peptides and the free amino acids in EDTA-soluble fraction B*

The numbers at the top of the columns refer to Scheme 2. Values are expressed as residues/1000 total amino acid residues.

		Fractions eluted from Dowex 50 (X8; H^+ form) resin column		
			Eluted with aq. NH_3	
	Total amino acid composition (1)	Peptides eluted with water (2)	Peptides (3)	Free amino acids (4)
Cystine (half)*	< 1	2	—	—
3-Hydroxyproline	—	—	—	—
4-Hydroxyproline	—	—	—	—
Aspartic acid	85	122	117	1
Threonine	31	40	61	21
Serine	64	92	107	30
Glutamic acid	129	203	195	10
Proline	28	18	75	34
Glycine	511	420	188	737
Alanine	58	33	95	144
Valine	27	25	—	—
Methionine†	10	18	13	5
Isoleucine	‡	‡	‡	‡
Leucine	34	‡	‡	‡
Tyrosine	4	3	—	—
Phenylalanine	8	5	19	11
Hydroxylysine	—	—	—	—
Lysine	8	9	72	3
Histidine	Trace	3	27	2
Arginine	3	8	33	1

* Recovered as cysteic acid.

† Recovered as methionine and methionine sulphoxide.

‡ In some cases in which no values are given, the chromatographic peak in the region that ordinarily corresponded to these amino acids was broadened and asymmetric and had a different E_{570}/E_{440} ratio. Further hydrolysis of these samples at 115° for 24 hr. in 6N-HCl did not change these properties.

Table 3. *Amino acid compositions of the hydrochloric acid-soluble components of the organic matrix of erupted bovine enamel*

The capital letters at the top of columns refer to fractions eluted from Sephadex G-25 (Scheme 3 and Fig. 3). Values are expressed as residues/1000 total amino acid residues.

	Fractions eluted from Sephadex G-25 column				
	A	B	C	D	E
Cystine (half)*	4	—	6	—	< 1
3-Hydroxyproline	Trace	—	—	—	—
4-Hydroxyproline	31	—	—	—	—
Aspartic acid	75	104	136	86	67
Threonine	60	47	50	42	32
Serine	74	81	111	117	133
Glutamic acid	104	168	224	176	113
Proline	119	100	26	19	20
Glycine	130	155	283	397	387
Alanine	110	65	65	77	72
Valine	59	46	24	23	29
Methionine†	30	14	1	3	2
Isoleucine	27	28	11	13	17
Leucine	49	63	22	28	31
Tyrosine	5	Trace	3	—	3
Phenylalanine	25	21	5	—	66
Hydroxylysine	27‡	—	3	—	—
Lysine	30	55	20	13	14
Histidine	17	27	8	7	13
Arginine	26	25	1	Trace	Trace

* Recovered as cysteic acid.

† Recovered as methionine and methionine sulphoxide.

‡ This chromatographic peak was more asymmetrical than the standard and may represent other components in addition to hydroxylysine.

Bio-Gel P–30, the major one of mol.wt. greater than 30000 and the smaller fraction of mol.wt. less than 30000. The small amount of material available and its relative insolubility prevented further physicochemical study.

The water-soluble non-diffusible fraction was found to contain approx. 20 μmoles of hexose/μmole of amino acid. This corresponds to a carbohydrate content of approx. 95% on a molar or residue basis. The fraction isolated from this material of mol.wt. greater than 30000, from its behaviour on gel filtration, also had a carbohydrate content of approx. 90–95%; the small amount of material of mol.wt. less than 30000 contained approx. 85–88% of carbohydrate. Qualitatively similar results were obtained when the amino acid content was computed on the basis of the recovery of amino acids from known weights of the samples.

Although thin-layer chromatography permitted only qualitative analysis of the carbohydrate components, it was obvious that glucose was by far the most predominant constituent of the hexoses identified in the total non-diffusible fraction, and in the higher-molecular-weight components. Smaller amounts of galactose, mannose and fucose were also present in these samples. Glucosamine was present in greater amounts than galactosamine in the whole non-diffusible fraction, but galactosamine was the predominant amino sugar in the higher-molecular-weight components. Only galactose could be identified in the lower-molecular-weight fraction, although this may have been due to the small size of the sample used.

The content of peptide-bound and free amino acids in fraction B of the EDTA-soluble material and their compositions (Scheme 2) are shown in Table 2. Except for amino acids with a strong negative charge such as free phosphoserine, free amino acids should be adsorbed on the Dowex 50 (H^+ form) resins at acid pH, and eluted at alkaline pH by aq. ammonia. This was confirmed by chromatographing mixtures of standard amino acids of acid hydrolysates of the enamel proteins on these resin columns. Therefore that portion of fraction B which was eluted with water from the Dowex 50 (H^+ form) resin should have theoretically been composed solely of peptide-bound amino acids. This was confirmed by chromatographing

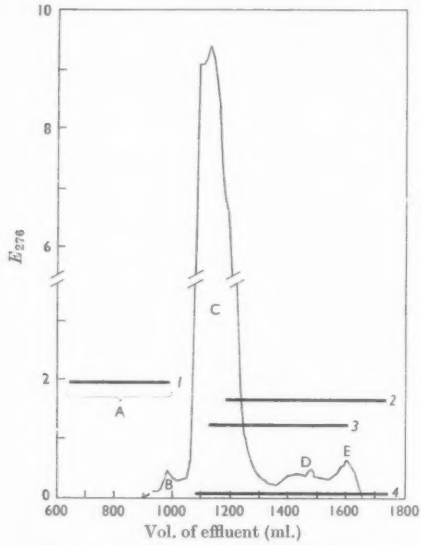


Fig. 3. Typical elution curve of the HCl-soluble peptides of erupted bovine enamel filtered through a column (3cm. × 170cm.) of Sephadex G-25 resin in 5% acetic acid, pH2·5, at 2°. The horizontal bars correspond to the positions of: *1*, bovine plasma albumin; *2*, amino acids (glycine and aspartic acid); *3*, peptides (mol.wt. 250–300); *4*, salt.

the water-eluted portion directly on the amino acid analyser. Only negligible quantities of free amino acids were identified.

In the fraction eluted from the Dowex 50 (X8; H^+ form) resin with aq. ammonia, approx. 25% of the amino acids was peptide-bound, the remainder existing as free amino acids. Of the entire amino acid content of fraction B, approx. 80% of the amino acids was peptide-bound.

Hydrochloric acid preparations. Table 3 summarizes the amino acid compositions of the fractions outlined in Scheme 3. Fig. 3 shows a typical elution curve of the hydrochloric acid-soluble fraction filtered through Sephadex G-25 resin. An insufficient amount of the hydrochloric acid-insoluble fraction was recovered for amino acid analysis.

Hydroxyproline was detected only in fraction A. The compositions of peptide-bound and free amino acids in fractions C and D are given in Tables 4 and 5 respectively. Approx. 90% of the amino acids in the aq.-ammonia-eluted portion of both fractions C and D existed as free amino acids, and only 10% or less was peptide-bound. In the entire fraction C, approx. 15% of the amino acids was peptide-bound, and in fraction D 42% of the amino acids was peptide-bound.

DISCUSSION

Many factors combine to make a study of the organic matrix constituents of mature dental enamel difficult. Because of the extremely small amount of protein present in the mature enamel (approx. 0·06% in bovine species) (Glimcher *et al.* 1964c), especially in relation to the adjacent layers of dentine and coronal cementum, which contain approx. 25–30% collagen, very many carefully prepared teeth must be utilized. The use of samples that are either of inadequate size, or not completely free of dentine or cementum, combined with extensive dialysis of material that is largely diffusible, has in the past resulted in either the recovery of insufficient amounts of material for even a single amino acid analysis (Piez, 1962), or the recovery of collagen-like proteins, presumably from the adjacent tissue layers (Hess, Lee & Neidig, 1953; Stack, 1954; Battistone & Burnett, 1956; Rodriguez & Hess, 1963).

The mild demineralization of the enamel of large numbers of teeth in turn requires the use of relatively large volumes of concentrated EDTA. Since most of the matrix constituents are soluble in EDTA and are diffusible, the recovery of small amounts of proteins and peptides from large volumes of concentrated EDTA, by methods that minimize denaturation and peptide-bond hydrolysis, presents many technical problems. To diminish the volume of fluid required, an alternative procedure to the EDTA method was tried. Decalcification of the enamel and solubilization of the organic matrix was carried out by continuously regenerating a fixed volume of fluid into which was dissolved hydrogen chloride gas to maintain the pH at 2·3–2·8. However, despite the low temperature employed (2°), the long time required for demineralization at pH2·3–2·8 resulted in peptide-bond hydrolysis, as evidenced by the marked increase in the percentage of the material existing as free amino acids compared with the samples demineralized in EDTA. Moreover, in contrast with the method employing EDTA in which contaminating collagen fibrils were not solubilized but recovered in the EDTA-insoluble precipitate, a small amount of collagen was dissolved in the hydrochloric acid and recovered in the fraction excluded from the Sephadex G-25 column together with the higher-molecular-weight components of the enamel matrix. In the procedures employing EDTA at slightly alkaline pH it was difficult to deal with the large volumes of solution directly, either by dialysis or by Sephadex-gel filtration, so that repeated acidification to pH1·5 was required to precipitate the EDTA and to permit concentration of the solution. To assess the effects of this acidification on the enamel peptides and proteins, a sample

Table 4. *Amino acid compositions of the peptides and free amino acids of hydrochloric acid-soluble fraction C*

The numbers at the top of the columns identify the subfractions as in Scheme 2 for the EDTA-soluble fraction B. Values are expressed as residues/1000 total amino acid residues.

		Fractions eluted from Dowex 50 (X8; H^+ form) resin column		
			Eluted with aq. NH_3	
	Total amino acid composition (1)	Peptides eluted with water (2)	Peptides (3)	Free amino acids (4)
Cystine (half)*	6	—	—	5
3-Hydroxyproline	—	—	—	—
4-Hydroxyproline	—	—	—	—
Aspartic acid	136	65	152	133
Threonine	50	25	—	64
Serine	111	96	91	125
Glutamic acid	224	432	113	237
Proline	26	31	171	10
Glycine	283	232	136	290
Alanine	65	41	82	61
Valine	24	20	46	17
Methionine†	1	10	—	4
Isoleucine	11	7	24	8
Leucine	22	20	65	16
Tyrosine	3	6	19	—
Phenylalanine	5	8	—	3
Hydroxylysine	3	—	Trace	—
Lysine	20	5	52	22
Histidine	8	2	52	6
Arginine	1	1	—	—

* Recovered as cysteic acid.
† Recovered as methionine and methionine sulphoxide.

of the EDTA solution was filtered through Sephadex G-25 without prior acidification. The elution pattern was qualitatively similar to that obtained from EDTA solutions that had been acidified, although the fraction retarded on the Sephadex G-25 column contained fewer free amino acids and somewhat more peptide-bound amino acids: approx. 85–90% of the amino acids was peptide-bound compared with 80% in the samples that were repeatedly acidified. Considering the small sample size used, this small difference could have been due to experimental error. Moreover, the peptides glycyl-L-aspartic acid and glycyl-L-leucylglycine chromatographed on the Dowex 50 (X8; H^+ form) resin were not hydrolysed into free amino acids. Since the major features that characterized the EDTA-soluble (and acidified) organic constituents were confirmed on samples that were not acidified, and since it is unlikely that peptide bonds were hydrolysed on the Dowex 50 (X8; H^+ form) resin, it is safe to conclude that the major constituents of the organic matrix are peptides of relatively low molecular weight and are readily diffusible.

The non-diffusible proteins and peptides, including a fraction whose mol.wt. was greater than 30000 as evidenced by its behaviour in gel filtration, are largely carbohydrate in nature (glycoproteins and glycopeptides) and contain both hexoses and hexosamines. They appear to be somewhat unusual with respect to their relatively high content of glucose. Galactosamine had been previously identified in the organic matrices of embryonic bovine enamel (Glimcher *et al.* 1961).

All the protein and peptide fractions were characterized by their relatively high concentrations of serine. The other amino acids present in significant amounts in the peptides were glycine, glutamic acid and aspartic acid. Glycine was the predominant amino acid present in the small peptides and as the free amino acid.

In several of the serine-rich peptides, partial acid hydrolysis and subsequent chromatography and electrophoresis revealed the presence of phosphoserine, which had previously been identified in the proteins of both embryonic and adult bovine enamel (Glimcher & Krane, 1964). The predominant

Table 5. *Amino acid compositions of the peptides and free amino acids of the hydrochloric acid-soluble fraction D*

The numbers at the top of the columns identify the subfractions as in Scheme 2 for the EDTA-soluble fraction B. Values are expressed as residues/1000 total amino acid residues.

		Fractions eluted from Dowex 50 (X8; H^+ form) resin column		
			Eluted with aq. NH_3	
	Total amino acid composition (1)	Peptides eluted with water (2)	Peptides (3)	Free amino acids (4)
Cystine (half)*	—	—	—	—
3-Hydroxyproline	—	—	—	—
4-Hydroxyproline	—	—	—	—
Aspartic acid	86	139	98	33
Threonine	42	11	——	60
Serine	117	83	29	138
Glutamic acid	176	117	—	216
Proline	19	30	29	8
Glycine	397	440	337	402
Alanine	77	38	73	69
Valine	23	17	39	20
Methionine†	3	4	20	—
Isoleucine	13	13	59	10
Leucine	28	24	170	27
Tyrosine	—	9	93	—
Phenylalanine	—	19	93	—
Hydroxylysine	—	—	—	—
Lysine	13	32	20	10
Histidine	7	21	15	7
Arginine	Trace	4	—	Trace

* Recovered as cysteic acid.
† Recovered as methionine and methionine sulphoxide.

amino acids noted in these peptides and the ones isolated in this study (aspartic acid, glutamic acid and glycine) have also been identified in the phosphate-containing peptides from collagen (Glimcher, Francois, Richards & Krane, 1964*a*), fibrinogen (Blombäck, Blombäck, & Searle, 1963), ovalbumin (Flavin, 1954); Perlmann, 1952), casein (Mellander, 1963) and calf-intestinal alkaline phosphatase (Engström, 1964). The persistence of serine-containing proteins and peptides in the enamel matrix during maturation, when over 99% of the protein constituents is lost (Glimcher *et al.* 1964*b*,*c*), may be due to the strong interaction between the serine-bound organic phosphate and the inorganic crystals (Glimcher & Krane, 1964).

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The Choline Acetyltransferase of Human Placenta

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1. Various methods for the extraction of choline acetyltransferase (acetyl-CoA–choline *O*-acetyltransferase, EC 2.3.1.6) from immature human placenta (18–28 weeks of gestation) are described. 2. The crude enzyme was found to be stable at $-18°$ and $+4°$ under a variety of conditions. 3. Purification methods, including ammonium sulphate fractionation, gel filtration on various grades of Sephadex and DEAE-Sephadex fractionation, have yielded a preparation of high specific activity.

The role of ACh* in the nervous system has prompted a considerable amount of research into the localization, isolation and properties of choline acetyltransferase, the enzyme responsible for ACh synthesis (for reviews see Hebb, 1957, 1963). These investigations have been concerned almost exclusively with the enzyme obtained from nervous tissue and, although it has been purified to some extent (Berman, Wilson & Nachmansohn, 1953; Ebashi & Kumagai, 1954; Burgen, Burke & Desbarats-Schonbaum, 1956; Mehrotra, 1961), the enzyme has never been isolated in the pure state. Possibly the most active non-mammalian source of choline acetyltransferase is the blowfly brain (Smallman, 1956), which, in crude form, has been calculated (Hebb, 1957) to have a specific activity 0·3 μmole of ACh produced/min./mg. of protein. Crude extracts of squid-head ganglion have a specific activity about 0·1 and Berman *et al.* (1953) achieved a tenfold purification of this material. The highest specific activity so far reported from mammalian tissue is about 0·05 (Ebashi & Kumagai, 1954), obtained by partial purification of the enzyme from guinea-pig brain.

The presence of ACh in the human placenta has been known for many years (Chang & Gaddum, 1933), and the presence of choline acetyltransferase has been confirmed by Comline (1946). The concentration of the enzyme reaches a maximum between 18 and 24 weeks of gestation, the specific activity being then about fourfold that of the most active mammalian nervous tissue (Hebb & Ratkovič, 1962). A study of the extraction, properties and purification of the placental enzyme and a comparison of its specificity with the enzyme of nervous origin was of obvious interest. Choline acetyltransferase of low specific activity isolated from full-term placenta has been described by Kato (1960).

A comparative specificity study of the partially purified enzyme has appeared (Hemsworth & Morris, 1964) and the enzyme's molecular weight has been estimated (Bull, Feinstein & Morris, 1964). Although the pure enzyme has not yet been isolated, a preparation of high specific activity has been obtained and the present paper describes the extraction and properties of crude choline acetyltransferase from 18–28-week human placentae, together with a comparative investigation of purification methods with particular reference to ammonium sulphate purification and gel filtration.

* Abbreviation: ACh, acetylcholine.

EXPERIMENTAL

Materials

CoA was prepared from baker's yeast by the method of Hebb (1955); for details see Nordenfelt (1963). The preparation contained 300–400 Lipmann units/ml. Phosphotransacetylase (acetyl-CoA–orthophosphate acetyltransferase, EC 2.3.1.8) was prepared from dried cells of *Clostridium kluyverii* (Worthington Biochemical Corp., Freehold, N.J., U.S.A.) according to instructions given by the Sigma Chemical Co., St Louis, Mo., U.S.A. (see Nordenfelt, 1963). Acetyl phosphate was either obtained from Worthington Biochemical Corp. or synthesized by the method of Avison (1955). Thioglycollic acid (Hopkin and Williams Ltd., Chadwell Heath, Essex) was redistilled *in vacuo* under N_2 and stored at $-10°$. All other chemicals were either of AnalaR quality or of the highest purity commercially available.

Solutions. Solution A: 0·05M-cysteine, neutralized to pH 6·4 with N-KOH, in 0·154M-NaCl. Solution B: 0·05M-thioglycollic acid, neutralized to pH 6·4 with N-KOH, in 0·154 M-NaCl. Solution C: 0·02M-sodium phosphate buffer,

pH6·6, containing 0·1M-NaCl, 5mM-EDTA and 5mM-thioglycollic acid, neutralized to pH6·6 with N-KOH.

Estimation of choline-acetyltransferase activity

Incubation. The incubation medium contained a coupled enzyme system, similar to that described by Hebb, Krnjević & Silver (1964), with phosphotransacetylase, CoA and acetyl phosphate as an enzymic source of acetyl-CoA. The complete medium contained, in final volume 0·9ml.: choline chloride, 11·5μmoles; KCl, 160μmoles; acetyl phosphate, 9·2μmoles; $MgCl_2$, 4·9μmoles; L-cysteine–HCl, 23μmoles, neutralized to pH6·4 with N-KOH; eserine sulphate, 0·13μmole; sodium phosphate buffer, pH6·9, 12·8μmoles; CoA, 20–30 units; phosphotransacetylase, 0·25mg.; the choline-acetyltransferase preparation (0·1ml.).

The medium was incubated at 39° for 10min. to allow formation of acetyl-CoA and the choline acetyltransferase was then added. After a further period at 39° (usually 1hr., but 10min. with very active preparations) the incubation was stopped by the addition of 6 drops of Universal Indicator (British Drug Houses Ltd.) and 0·3N-HCl (0·5ml.). The acidified incubation mixture was quantitatively transferred to a 50ml. conical flask with 3×2ml. portions of frog Ringer solution and brought to 100° to destroy excess of acetyl phosphate. After cooling to room temperature, 0·5% (w/v) Na_2HPO_4 (1ml.) was added and the volume was adjusted to 20 or 40ml. with frog Ringer solution. The final pH was approximately pH4. The activity of the acetyl-CoA-generating system was checked at each incubation with standard rabbit-brain choline acetyltransferase prepared as an acetone-dried powder extract in solution A. The incubation medium (minus the choline acetyltransferase) could, for convenience, be stored at −18° for periods up to 4 weeks without loss of activity.

The range of enzyme concentrations over which the substrates used above produced a linear rate of ACh synthesis was determined by incubation of various dilutions of a highly active preparation. For subsequent estimation of unknown enzyme activity, preparations were diluted so that the rate of synthesis of ACh was within the linear range.

Assay of acetylcholine. The incubation mixture, prepared as described above, was assayed on the eserinized frog rectus abdominis muscle according to the procedure of Feldberg (1950), with the same precautions to control the effect of sensitizing substances on the rectus muscle. In addition (Feldberg, 1950), it was sometimes convenient to use a heat-treated inactivated enzyme for control incubations. All determinations were made in duplicate. Overall errors of estimation were computed to be within ±10% but duplicates rarely differed by more than ±5%.

Enzyme units. One unit of enzyme activity was defined as 1μmole of ACh synthesized/min. at 39° (m-units were used when appropriate). Specific activity was expressed as units/g. of placental tissue, units/g. of acetone-dried powder, units/ml. of extract or units/mg. of protein.

Protein. The protein content in column eluates was estimated by absorption at 280mμ (1mm. cells, Unicam SP.500 spectrophotometer). For measurements of specific activity in terms of protein concentration the ratio of the extinctions at 280 and 260mμ (Warburg & Christian, 1941) was measured or protein N was determined by the micro-Kjeldahl procedure.

Extraction of tissue

Placentae. Immature human placentae of 18–28 weeks gestation, produced either by spontaneous abortion or by clinical termination of pregnancy, were obtained as fresh as possible from the Maternity Hospital, Mill Road, Cambridge. If extensive blood clotting had not occurred the placentae were perfused with cold 0·154M-NaCl through the umbilical cord vessels until the tissue was cleared of as much blood as possible. The cord was removed and the adjacent chorionic and amnionic membranes were gently teased away from the required pulpy tissue. The tissue was usually processed immediately but could be stored at 0° for several days without appreciable loss of activity.

Extraction procedures. On a small scale, acetone-dried powders or homogenates were prepared. Acetone-dried powders were prepared as described by Hemsworth & Morris (1964) for brain tissue. The powders were extracted at a concentration of 100 or 50mg. of powder/ml. with cold 0·154M-NaCl either alone or in combination with other reagents added to study their effect on enzyme activity. The extracts were centrifuged (International type SB, rotor 225, 1380*g* for 10min.) and the supernatants were either stored at −18° or examined immediately for enzyme activity.

Homogenates were prepared from chopped tissue with cold solution B at a concentration of 100mg. of tissue/ml. in a hand-operated ball-type glass homogenizer. The homogenate was centrifuged as described above before estimation.

For extraction of whole placentae, where the weight of tissue was 100g. or more, the tissue was chopped and then homogenized at 4° in a Townson and Mercer top-drive macerator for 1min. with the aid of a sufficient volume (usually about 100ml.) of cold solution B to form a free-flowing slurry. The latter was then freeze-dried in the cold room (4°) and the resulting powder was extracted with solution B and centrifuged (Servall angle-head centrifuge, 12000*g* for 15min.). The centrifugate was re-extracted with sufficient solution B to give a final concentration 75mg. of powder/ml. and centrifuged again. The combined supernatants were stored at −18° immediately, or, in the later part of this investigation, subjected to fractionation with $(NH_4)_2SO_4$ between 15 and 30% (w/v) before storage. This method of extraction gave fairly reproducible activities from a large number of placentae.

Purification of enzyme

Ammonium sulphate fractionation. Preliminary experiments showed that at pH values much below 5 considerable losses of enzyme activity occurred and therefore salt fractionation below this pH was not studied. Extracts of freeze-dried tissue in solution B were adjusted to pH5, 6 and 7 respectively, and were progressively fractionated with $(NH_4)_2SO_4$ in 5% (w/v) steps between 15 and 30%. The pH was readjusted where necessary with 0·5N-acetic acid or 0·5N-KOH. Precipitation at each step was allowed to proceed for 1hr. at 4° before centrifugation (12000*g* for 15min.). The supernatants at each stage were sampled before further addition of $(NH_4)_2SO_4$, the precipitates resuspended in solution B and centrifuged; the insoluble material was discarded. After dialysis overnight at 4° against 3×2l. changes of solution B the samples were

recentrifuged and the supernatants then assayed for choline-acetyltransferase activity and protein content.

The effect of refractionation of enzyme preparations obtained by an initial 20–30% (w/v) $(NH_4)_2SO_4$ step at pH 5 was also studied. The precipitates and supernatants were treated as described above.

Gel filtration. The various grades of Sephadex were allowed to swell in 0·154 M-NaCl for periods from 1 day for Sephadex G-25 to 1 week for the most porous gel, Sephadex G-200. During this time 'fines' were removed by decantation. The gel beds were formed by allowing a slurry of gel particles to percolate under gravity into vertical glass chromatography columns fitted with porosity 1 sintered-glass disks and previously filled with 0·154 M-NaCl. After 2–3 cm. of gel had settled the excess of liquid was allowed to percolate through the lengthening gel bed. When the required bed height was obtained 0·154 M-NaCl was percolated continuously through the column in the cold room. Before an experiment the columns were equilibrated with the eluting solvent (either solution B or solution C). Percolation of high-molecular-weight blue-dyed dextran (Pharmacia) through the columns prepared in this way produced compact and even bands and enabled the void volumes to be determined. For Sephadex G-75 gel filtrations two columns of dimensions 45 cm. × 1·7 cm. and 80 cm. × 1·7 cm. were employed to investigate the effect of column length on the efficiency of separation. Subsequently columns of dimensions 102 cm. × 4 cm. were used for Sephadex G-100 and G-200 filtration experiments.

The flow rates obtained with Sephadex G-75 and G-100, employing a small hydrostatic head, were about 13 and 3·2 ml./hr./cm.^{2} respectively. It was found necessary, with Sephadex G-200 columns, to form a 3 cm. layer of coarse Sephadex G-25 above the sintered disk before preparing the main Sephadex G-200 bed. This technique prevented the sinter from being progressively blocked by Sephadex G-200 gel particles and a flow rate, approximately constant over several experiments, of 1·6 ml./hr./cm.^{2} was obtained.

Enzyme solutions were applied directly to the drained surface of Sephadex G-75 gel columns. With the more porous gels a 3 cm. layer of Sephadex G-25 was formed on the top of the column and the enzyme preparation was carefully layered on to the gel surface under the buffer so as to give a very compact starting band. Where necessary, the density of the enzyme solution was adjusted by the addition of sucrose. Column eluates were collected in a Locarte fraction-collector with a constant-volume collecting device. Samples of the fractions were analysed for their protein and choline-acetyltransferase content and the remainder of the fractions were stored at temperatures below 0° (−10° or −18°). The active material was then thawed to 0° and concentrated.

Concentration of column eluates. A number of techniques including freeze-drying, pervaporation, addition of dry Sephadex G-25, Carbowax, $(NH_4)_2SO_4$ precipitation and ultrafiltration were investigated. The best methods were found to be ultrafiltration at 0° for enzyme preparations of intermediate specific activity and $(NH_4)_2SO_4$ precipitation (0–30%, w/v) at 4° for material of high specific activity. Any $(NH_4)_2SO_4$ which remained in the redissolved precipitate was removed by passage of the solution through a column (15 cm. × 1 cm.) of Sephadex G-25 equilibrated with solution C. The enzyme preparation was then stored at −70° or used immediately for further purification.

DEAE-Sephadex. This medium was used to purify further the enzyme obtained from Sephadex G-200 columns. The gel powder was allowed to swell in 0·1 M-NaCl for 4 days, during which time the 'fines' were removed. The suspension was titrated to pH 6·6 with 0·2 M-sodium phosphate buffer, pH 6·6, and then equilibrated with solution C. The enzyme preparation (usually 5 ml.) from the Sephadex G-200 step, equilibrated with solution C, was applied to a column (15 cm. × 1 cm.) of the DEAE-Sephadex and eluted with the same solution. Fractions (10 ml.) were collected and analysed as before. Active fractions were concentrated by $(NH_4)_2SO_4$ precipitation and stored at −70°.

Immunoelectrophoresis. A modification of the technique of Grabar & Burtin (1960) was employed, 1·5% (w/v) agar gel containing 0·1% (w/v) of sodium azide and veronal buffer, pH 8·6 and I 0·025, being used. The buffer vessels contained the same buffer but with I 0·05. The electrophoresis was carried out for 2 hr. at 7 v/cm. Longitudinal slots were then cut along the migration axis and anti-human serum was placed in them. After diffusion overnight at room temperature the plates were examined for precipitin lines caused by the presence of serum proteins in the choline-acetyltransferase preparation. This technique was used in the later stages of the purification to follow the removal of contaminating serum proteins.

RESULTS

Incubation and assay. The incubation medium used in these experiments was designed originally for the determination of the small amounts of choline acetyltransferase from nervous tissue, but it will be seen from Fig. 1 that in the present

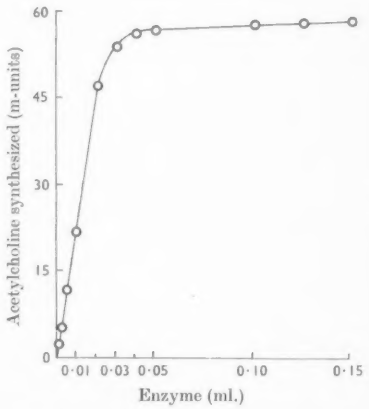

Fig. 1. Production of ACh by standard incubation medium. Various dilutions of a placental extract, partially purified by fractionation between 15 and 30% (w/v) $(NH_4)_2SO_4$, were incubated for 1 hr. at 39°. The ACh produced was determined by bioassay. For details see the Experimental section.

experiments a linear relation between enzyme concentration and ACh synthesis was obtained up to 46 m-units of ACh/min. At intermediate values of specific activity the incubation medium was capable of a substantially linear ACh production for incubation periods up to 60 min. but for preparations of specific activity higher than 600 mμmoles/min./mg. of protein it was both necessary and convenient to incubate for 10 min. only.

Extraction procedures. The enzyme in homogenates of placental tissue had a higher specific activity than did extracts of acetone-dried powders, which were also more variable in their activity. Thus the specific activities of homogenates of two 25-week placentae were 1·25 and 1·07 units/g. of tissue, whereas the corresponding acetone-dried-powder activities were 0·80 and 0·37 unit/g. of tissue respectively. Control experiments showed that freeze-drying of homogenized placental tissue did not lead to loss of activity.

Table 1. *Stability of crude choline acetyltransferase at* $-18°$

Placental tissue, acetone-dried powders and various extracts of acetone-dried powders were stored at $-18°$ over a period of 2 months and examined periodically for enzyme activity. Tissue was stored in stoppered test tubes and acetone-dried powders were stored in evacuated Thunberg tubes containing P_2O_5. The acetone-dried-powder extracts (50 mg. of powder/ml.) were prepared by extraction with cold 0·154 M-NaCl alone or in the presence of 0·05 M-cysteine, pH 6·4, or 0·05 M-choline. For estimation of enzyme activity, tissue samples were homogenized in cysteine in NaCl (100 mg. of tissue/ml.) and the acetone-dried powders were extracted with cysteine in NaCl (50 mg. of tissue/ml.). For enzyme estimation tissue homogenates were diluted 1:1 (v/v) with 0·154 M-NaCl; all other preparations were diluted 1:10 (v/v). For details of enzyme estimation see the text. Enzyme activity: 1 unit = 1 μmole of ACh synthesized/min. at 39°.

	Enzyme activity (units/g. of tissue)				
	Zero time	2 weeks	4 weeks	6 weeks	8 weeks
Tissue	1·07	1·05	1·03	1·31	1·18
Acetone-dried powder	0·36	0·37	0·40	0·38	0·25
NaCl extract	0·34	0·40	0·38	0·34	0·34
Cysteine–NaCl extract	0·43	0·48	0·43	0·39	0·43
Choline–NaCl extract	0·34	0·44	0·39	0·39	0·39

Table 2. *Effect of various reagents on the choline-acetyltransferase activity of placental acetone-dried-powder extracts at* 4°

Various extracts of placental acetone-dried powder (50 mg. of powder/ml. of extractant) were prepared at 4° with 0·154 M-NaCl alone, with 0·05 M-EDTA in NaCl and with 0·05 M-EDTA in NaCl containing 50 or 100 mM-cysteine, -thioglycollate, -2-mercaptoethanol, -2,3-dimercaptopropan-1-ol or -choline respectively. The solutions were adjusted to pH 6·4 before the acetone-dried powders were extracted. Samples (0·1 ml.) were withdrawn at various times, diluted 1:10 (v/v) with 0·154 M-NaCl and the choline-acetyltransferase activity was determined as described in the text. 1 m-unit = 1 mμmole of ACh synthesized/min. at 39°. The molarities under the heading 'Concn.' in the Table refer to the final concentration of the reagents added to the EDTA in NaCl solution.

		Enzyme activity				
		Initial (m-units/ml.)	Residual (% of initial)			
Extraction medium	Concn. (mM)		8 days	14 days	21 days	28 days
NaCl	zero	70	95	110	89	95
EDTA–NaCl	zero	81	82	67	35	27
Cysteine–EDTA–NaCl	50	81	104	104	123	111
	100	81	109	109	96	96
Thioglycollate–EDTA–NaCl	50	81	114	109	122	96
	100	81	110	107	105	96
2-Mercaptoethanol–EDTA–NaCl	50	88	100	92	83	71
	100	88	100	96	45	27
2,3-Dimercaptopropanol–EDTA–NaCl	50	81	91	50	50	30
	100	81	82	25	26	33
Choline–EDTA–NaCl	50	81	100	87	85	64
	100	81	109	82	59	61

Stability of stored crude enzyme preparations. Tables 1 and 2 show that the crude enzyme, stored under a variety of conditions at −18° and +4°, was generally very stable but the presence of 0·1 M-mercaptoethanol or 2,3-dimercaptopropanol at both concentrations employed caused serious activity losses. The presence of 0·05 M-EDTA caused a considerable loss of activity in the absence of any thiol-protecting agent (Table 2) but this loss was partially prevented by choline.

Stability at 39°. The effects of incubation of the unpurified enzyme at 39° for 2 hr. under various conditions are shown in Table 3. This experiment was designed to accelerate inactivation of the enzyme and it will be seen that in the absence of cysteine and EDTA all activity was lost. These reagents present separately provided some protection but nearly 70% of the initial activity was preserved when both were present.

Fractionation with ammonium sulphate. The results are shown in Fig. 2. At all three pH values the highest specific activities were obtained in the protein which precipitated between 20 and 25% (w/v) ammonium sulphate. The most active material, however, was obtained at pH 5·0 and the least active at pH 7·0. Furthermore, the overall recovery at pH 5·0 was 70% whereas at pH 6·0 and pH 7·0 the recoveries were 57% and 50% respectively. At pH 5·0 relatively more enzyme was precipitated at 20% than at 30% whereas the reverse was found at the higher pH values.

The effect of refractionation of enzyme initially precipitated between 20 and 30% (w/v) ammonium sulphate at pH 5·0 is shown in Table 4. In the small-scale experiment (*a*), rather more than a 20-fold increase of specific activity was obtained in the 26–

Table 3. *Effect of EDTA on choline-acetyltransferase activity at* 39°

Acetone-dried powders of placental tissue were extracted with 0·05 M-cysteine, pH 6·5, in 0·154 M-NaCl or in 0·154 M-NaCl alone, both at a concentration of 50 mg. of powder/ml. After dilution (1:1, v/v) with 0·154 M-NaCl or 0·10 M-EDTA, pH 6·5, the extracts were incubated at 39° for 2 hr.; control samples were kept at 0° during this period. For estimation of choline-acetyltransferase activity 0·1 ml. samples were incubated with the substrate medium as described in the text. 1 m-unit = 1 mμmole of ACh synthesized/min. at 39°.

Extract	Choline-acetyltransferase activity (m-units/ml. of extract)	
	Control (0°)	At 39°
NaCl	18·7	0
EDTA–NaCl	16·0	5·17
Cysteine–NaCl	46·0	5·17
Cysteine–EDTA–NaCl	38·4	26·2

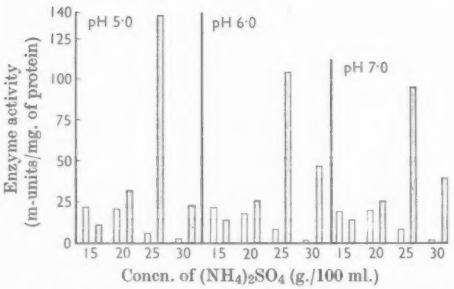

Fig. 2. Effect of pH on $(NH_4)_2SO_4$ fractionation. Extracts of freeze-dried tissue in solution B (940 ml., 17 mg. of protein/ml.) were adjusted to pH 5, 6 and 7 respectively and fractionated with $(NH_4)_2SO_4$ in 5% steps from 15 to 30% (w/v) at 4°. The pH was readjusted where necessary with 0·5 N-acetic acid or 0·5 N-KOH. Precipitation at each step was allowed to proceed for 1 hr. before centrifugation (12000*g* for 15 min.). The supernatants at each stage were sampled before further addition of $(NH_4)_2SO_4$, and the precipitates resuspended in solution B and centrifuged; the insoluble material was discarded. All solutions were dialysed overnight at 4° against three changes of solution B, recentrifuged and the supernatants then assayed for choline-acetyltransferase activity (see the text) and protein N (micro-Kjeldahl). Enzyme activity: 1 m-unit equals 1 mμmole of ACh synthesized/min. at 39°. Specific activities: □, supernatants; ■, resuspended precipitates.

Table 4. *Refractionation of choline acetyltransferase with ammonium sulphate at* pH 5·0

Two enzyme preparations [(*a*) 25 ml., specific activity 130 m-units/mg. of protein, 3·8 mg. of protein/ml.; (*b*) 105 ml., specific activity 152 m-units/mg. of protein, 4·5 mg. of protein/ml.] obtained from an initial precipitation between 20 and 30% (w/v) $(NH_4)_2SO_4$ at pH 5·0 were refractionated in 2% (w/v) steps at pH 5·0. The precipitates were extracted with 0·05 M-thioglycollate, pH 6·5, in 0·154 M-NaCl, dialysed at 4° against three changes of the same solution and their choline-acetyltransferase activity and protein content were determined (see the text). Enzyme activity: 1 m-unit = 1 mμmole of ACh synthesized/min. at 39°.

$(NH_4)_2SO_4$ fraction (%, w/v)	Choline-acetyltransferase specific activity (m-units/mg. of protein)	
	(*a*)	(*b*)
0–20	—	83
0–22	150	—
20–22	—	277
22–24	100	394
24–26	351	347
26–28	390	70
28–30	18	8

28% fraction, whereas in the larger-scale experiment (*b*), a similar degree of purification was found in the 22–24% fraction. Enzyme preparations with a specific activity about 300 m-units/mg. of protein, obtained by the two-stage ammonium sulphate procedure, were very unstable and sometimes lost up to 50% of their activity in 2 weeks at $-18°$. Subsequent investigation indicated that preparations subjected to the first 15–30% (w/v) ammonium sulphate stage only were much more stable and could be stored at $-18°$ without significant loss for several months. As a routine, placentae were therefore processed only to this stage before subjection to gel filtration.

Gel filtration

Fractionation on Sephadex G-75. The protein, enzyme-activity and specific-activity elution patterns resulting from (*a*), fractionation on a 45 cm. × 1·7 cm. column, and (*b*), refractionation on an 80 cm. × 1·7 cm. column, are shown in Fig. 3. Activity was associated with the trailing edge of the excluded protein in Fig. 3(*a*) and the specific-activity peak was displaced slightly farther to the right with a maximum of 79 m-units/mg. of protein, representing a purification factor of 2·75. The active fractions were pooled, concentrated by freeze-drying and the powder, dissolved in 2 ml. of solution B, was refractionated on the larger column. Although the displacement of activity from excluded protein was not greatly improved a further increase of maximum specific activity to 173 m-units/mg. of protein was obtained, giving an overall purification factor of 6·1 for the two columns.

Fractionation on Sephadex G-100. In a preliminary experiment, with a small column (45 cm. × 1·7 cm.), the choline-acetyltransferase activity was eluted once again in the trailing edge of the excluded protein; the relevant portion of the elution diagram is shown in Fig. 4(*a*). The preparation applied to this column had a specific activity 0·23 unit/mg. of protein, obtained by Sephadex G-75 fractionation. The most active fractions from this column lost 50% of their activity in 4 days at $-10°$ but nevertheless a maximum specific activity 0·67 unit/mg. of protein was found in tube 17 subsequent to this loss. A typical elution diagram obtained from experiments with the larger column (102 cm. × 4 cm.) is shown in Fig. 4(*b*). A much clearer separation of activity from the excluded protein was found, illustrating the fact that column length is a very important factor in gel-filtration resolving power.

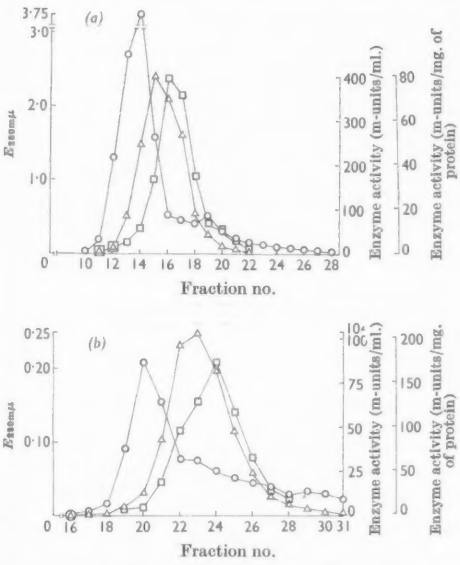

Fig. 3. Fractionation of choline acetyltransferase on Sephadex G-75. (*a*) Elution pattern given by $(NH_4)_2SO_4$-fractionated enzyme (2 ml., specific activity 28·4 m-units/mg. of protein). Column dimensions: 45 cm. × 1·7 cm. Fraction volume 5 ml. (*b*) Effect of refractionation of the concentrated (freeze-dried) activity (5 ml.) on a longer column (80 cm. × 1·7 cm.). For details of determination of enzyme activity see the text. Eluent: solution B. Fraction volume 5 ml. ○, E_{280}, 1 mm. cells; △, choline acetyltransferase (m-units/ml.); □, choline acetyltransferase (m-units/mg. of protein). 1 unit equals 1 μmole of ACh synthesized/min. at 39°.

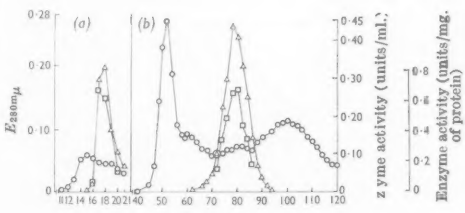

Fig. 4. Fractionation of choline acetyltransferase on Sephadex G-100. (*a*): Elution pattern of Sephadex G-75-purified material (5 ml.; 0·23 unit/mg. of protein). Column dimensions: 45 cm. × 1·7 cm. (*b*): Fractionation of $(NH_4)_2SO_4$-purified material (20 ml., 68·4 m-units/mg. of protein) on a large (102 cm. × 4 cm.) column. Eluent: solution B (see the text). Fraction volume 10 ml. ○, E_{280}, 1 mm. cells; △, choline acetyltransferase (m-units/ml.); □, choline acetyltransferase (m-units/mg. of protein). 1 unit equals 1 μmole of ACh synthesized/min. at 39°.

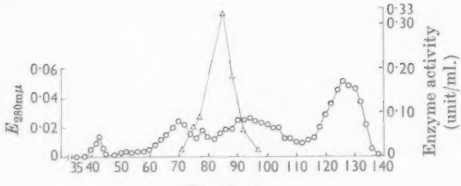

Fig. 5. Fractionation of choline acetyltransferase on Sephadex G-200. Elution pattern obtained from $(NH_4)_2SO_4$-purified material (24ml., 158m-units/mg. of protein). Column dimensions: 102cm. × 4cm. Eluent: solution C (see the text). Fraction volume 10ml. For determination of enzyme activity see the text. ○, E_{280}, 1mm. cells; △, choline acetyltransferase (unit/ml.). 1 unit equals 1μmole of ACh synthesized/min. at 39°.

The bulked activity (tubes 73–87) was 0·35 unit/mg. of protein and concentration of this material by precipitation with ammonium sulphate gave a further increase to 0·5. The recovery of activity was 70% and the concentrated material lost only 10% of its activity in 2 weeks at −18°. It will be noticed that material of similar specific activity prepared by the two-stage ammonium sulphate procedure was considerably less stable.

Fractionation on Sephadex G-200. The volume of enzyme preparation and the way in which it was applied to the column were critical. Volumes greater than 25ml. produced less separation of the major protein bands and inferior results were obtained if the applied enzyme layer was distorted or caused to diffuse during the layering procedure. The elution diagram of a satisfactory experiment is shown in Fig. 5. In this preparation there was very little protein of high molecular weight (> 200000) and the enzyme was eluted between two of the three main protein bands. The total activity eluted was about 90% and the combined peak tubes (74–90) contained 60% of the total. The ammonium sulphate-concentrated material had a specific activity 1·28 units/mg. of protein, representing an eightfold purification and an overall purification from crude extract of 48-fold. Similar results were obtained in several experiments; the highest specific activity achieved was 1·55 units/mg. of protein. In these experiments the enzyme activity was determined after 10min. incubation only.

Fractionation on DEAE-Sephadex. Immunoelectrophoresis and analytical ultracentrifugation of the active material derived from the Sephadex G-200 step revealed that the main contaminating proteins of serum origin were albumin and 7s globulin. Further experiments showed that human serum albumin was retained on columns of DEAE-Sephadex equilibrated with solution C, whereas choline acetyltransferase was not. A further twofold purification resulted when Sephadex G-200-fractionated enzyme was treated in this way. Thus a specific activity 2·34 units/mg. of protein was found in the first 10ml. fraction eluted from a column to which material with specific activity 1·28 was applied. Recoveries were about 50–70% and subsequent immunoelectrophoretic analysis revealed that the contaminating albumin had been removed. The presence of non-serum protein in the $\alpha\beta$-globulin region (probably containing the choline acetyltransferase) was revealed after plates, subjected to electrophoresis but not immunodiffusion, were stained with Amido Black.

DISCUSSION

The purification of choline acetyltransferase was undertaken to facilitate the study of the enzyme present in nervous tissue and the choice of the human placenta, a non-innervated organ, as source material may seem surprising. However, the placental and rabbit-brain enzymes, for instance, have similar molecular weights (Bull *et al.* 1964) and have a similar specificity both with regard to *N*-alkyl analogues of choline (Hemsworth & Morris, 1964) and to their capacity to synthesize propionyl- and butyryl-choline (D. Morris, unpublished work). Further, the high concentration of the enzyme in the immature placenta and the ease with which it is released from the subcellular structure (Hebb & Ratkovič, 1962) make the placenta suitable for the present purpose.

It will have been noticed that tissue homogenates (Table 1) had a much higher activity than did extracts of acetone-dried powders. This is believed to be due to the ease with which the enzyme is released from the tissue; activity is probably lost in the first acetone wash. Brain choline acetyltransferase, which is only released from its binding sites with some difficulty, does not exhibit this difference of activity between homogenates and acetone-dried powders.

The crude enzyme, although very stable under most conditions, lost activity in the presence of some thiol-protecting agents but not others (Table 2). It is conceivable that dimercaptopropanol and mercaptoethanol caused fission of disulphide bonds necessary for the maintenance of the integrity of the enzyme. Mercaptoethanol also caused inhibition when it was substituted for cysteine in the incubation medium.

The effects of EDTA on enzyme activity (Tables 2 and 3) suggest that one or more thiol groups are present in the active enzyme. At low temperatures (0° and 4°) the inhibitory effect of EDTA can be

attributed to the removal, by this reagent, of protective metal ions. At 39°, where inactivation may be due, partly at least, to oxidation of essential thiol groups, removal of cations by EDTA would prevent metal-ion catalysis of this oxidative process both in the enzyme itself and in the added cysteine. The overall effect would be retention of activity. The presence of essential thiol groups in the choline acetyltransferase of the squid-head ganglion has been postulated by Reisberg (1957).

The higher specific activities obtained by refractionation with ammonium sulphate at pH 5·0 after the initial 15–30% ammonium sulphate step were not very reproducible and the preparations were very unstable compared with material of similar activity produced by Sephadex G-100 gel filtration. In the experiments illustrated in Table 4, the starting protein concentrations were very similar, namely (*a*) 3·8mg. of protein/ml., (*b*) 4·5mg. of protein/ml., but the highest specific activity occurred at different concentrations of ammonium sulphate. The small difference in protein concentration may, however, be sufficient to account for the results (Dixon & Webb, 1964). The refractionation step is included in this paper as a useful and rapid procedure when preparations of intermediate specific activity are required for immediate use.

It is evident that columns of Sephadex G-75 can equilibrate with molecules of molecular weight greater than the 40000 originally claimed by the manufacturers. A provisional value for the molecular weight of placental choline acetyltransferase, determined by sedimentation only, of 59000 has been found (Bull *et al.* 1964). Fig. 3 shows that the enzyme is not excluded from the inside of Sephadex G-75 gel particles. The greatly improved separation found with Sephadex G-200 illustrates the fact that equilibration of molecules between the internal and external volumes of the gels is by no means an 'all-or-none' phenomenon. $S_{20,w}$ of the enzyme is 4·6s, a value very similar to that of albumin. However, the elution volume from Sephadex G-200 columns is appreciably lower than that of albumin and this observation, when considered in relation to the sedimentation data of the two proteins, suggests that the placental enzyme may have a higher molecular weight and a markedly greater degree of asymmetry than albumin. The provisional molecular weight of 59000 is therefore likely to be too low because of the asymmetry of the enzyme. It is of interest that the molecular weight of rabbit-brain choline acetyltransferase, calculated from sedimentation and diffusion data, is 67000 (Bull *et al.* 1964). The frictional ratio of this enzyme is calculated to be 1·17, a value less than that of albumin. It is possible therefore that the choline acetyltransferase of placenta and rabbit brain may differ appreciably in shape. There have been reports that with proteins of different frictional ratios the elution volumes obtained with Sephadex G-200 correlate more closely with diffusion coefficients than with molecular weight (Laurent & Killander, 1964; Siegel & Monty, 1965).

The major problems associated with the purification of placental choline acetyltransferase are the presence of large amounts of serum proteins and the instability of the partially purified enzyme. Immunoelectrophoresis has greatly facilitated the design of procedures for the separation of the enzyme from serum proteins and most of the latter have now been removed. The different degrees of stability found with enzyme of similar specific activity but prepared by different procedures suggest that other proteins present may affect stability. No protection was afforded by the addition of diisopropyl phosphorofluoridate (0·1mM).

In conclusion, a sequential procedure involving ammonium sulphate precipitation, Sephadex G-200 gel filtration and ion-exchange chromatography on DEAE-Sephadex has resulted in 80–100-fold purification of human placental choline acetyltransferase. Further studies are in progress which, it is hoped, will lead to the isolation of the pure enzyme.

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Enzymic Hydrolysis of Sphingomyelin by Rat Liver

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1. An enzyme that hydrolyses sphingomyelin to ceramide (*N*-acylsphingosine) and phosphorylcholine was isolated from rat liver. 2. The enzyme is particle-bound (mitochondria or lysosomes) and can be solubilized by ultrasonic treatment and freezing and thawing. 3. It has been partially purified by precipitation at pH 5·2, neutralization and ammonium sulphate fractionation. 4. The enzyme is activated by Triton X-100 (0·2%) or low concentrations of cetyltrimethylammonium bromide (0·02%), higher concentration being inhibitory. 5. The optimum pH is 5–5·5. 6. Of synthetic substrates tested, the *erythro* isomers of DL-*trans*-2-*N*-palmitoyl-1-*O*-phosphorylcholinesphingosine or dihydrosphingosine were hydrolysed at a rate similar to the natural compound. The *threo* isomer was hydrolysed much more slowly. The enzyme had little activity on lecithin. 7. The split products of the hydrolysis have little inhibitory effect.

The hydrolysis of sphingomyelin by enzymes derived from animal tissues has not been studied in detail. This is in contrast with the considerable information gained on the hydrolysis of lecithin and other glycerophosphatides (Dawson, 1962; Kates, 1960).

Enzymes that split sphingomyelin so far described belong to the group of phospholipases C (EC 3.1.4.3). These enzymes degrade sphingomyelin to phosphorylcholine and ceramide (*N*-acylsphingosine). They also split glycerophosphatides, producing diglyceride and a phosphoryl base. Phospholipases C were found mainly in bacteria and have been isolated from *Clostridium welchii* and *Bacillus cereus* (Kates, 1960). A specific sphingomyelin-hydrolysing enzyme has been identified in the strain $\alpha\beta$ of *Staphylococcus pyogenes* (Doery, Magnusson, Cheyne & Gulasekharam, 1963). The phospholipase C activity is calcium-dependent (Macfarlane & Knight, 1941). Ether accelerates considerably the rate of hydrolysis of the phosphatides (Hanahan & Vercamer, 1954). The purified enzyme from *C. welchii* is isoelectric at pH 5·5, and requires positively charged ions or positive amphipathic compounds for maximal activity (Bangham & Dawson, 1962). The existence of an enzyme in calf liver splitting sphingomyelin into ceramide, phosphoric acid and choline has been reported (Thannhauser & Reichel, 1940; Thannhauser & Boncoddo, 1946). A similar enzyme was described in pig spleen (Fujino, 1952). In both cases, activity was very low and prolonged incubation times were necessary to obtain significant hydrolysis. This paper deals with the isolation of an enzyme from rat liver which hydrolyses sphingomyelin (sphingomyelinase), its partial purification, properties and specificity. A preliminary account has been published (Heller & Shapiro, 1963).

EXPERIMENTAL

Materials

Sphingomyelin was prepared from various tissues, after extraction with chloroform and methanol according to Folch, Lees & Sloane-Stanley (1957). The lipid material, obtained after evaporation of the organic layer, was subjected to mild alkaline hydrolysis with aq. N-NaOH at 37° for 16–24 hr. with continuous shaking, according to Thannhauser, Benotti & Boncoddo (1946), or in methanolic N-NaOH for 2 hr. at 37°. After the hydrolysis, which affects only the glycerolipids, the sphingomyelin was purified by silicic acid column chromatography (Hanahan, 1960). The sphingomyelin thus obtained gave rise to two spots (depending on the fatty acid residue) when chromatographed on thin layers of silicic acid according to Mangold (1961). Analysis of the sphingomyelin constituents according to Sweeley (1963), and to Böttcher, Preis & Vant Gent (1961), gave the following ratios: choline/P, 1·0; sphingosine/P, 0·77; amide bond/P, 0·9.

The synthetic sphingomyelins, *N*-palmitoyl derivatives of D-*erythro*-sphingomyelin and DL-*erythro*-dihydrosphingomyelin, and DL-*threo*-dihydrosphingomyelin, were a gift from Professor D. Shapiro of the Weitzman Institute, Rehovoth, Israel.

Lecithin was prepared from egg yolk according to Pangborn (1951), or from rat livers by silicic acid column chromatography (Hanahan, 1960). Its purity was confirmed by thin-layer chromatography and by ester/P ratio. Aqueous suspensions of the phosphatides were prepared by ultrasonic treatment at 20 kcyc./sec. (Branson Instruments

Inc.) for several minutes at 0–4°. The suspensions were stable when kept in glass-stoppered tubes in the refrigerator at concentrations below 0·01 M.

Phosphatidic acid was prepared from egg lecithin by the action of phospholipase D (EC 3.1.4.4) according to Kates (1956).

Phosphorylcholine and *p*-nitrophenyl phosphate were purchased from the Sigma Chemical Co. (St Louis, Mo., U.S.A.). Triton X-100 was obtained from City Chemical Corp. (New York, N.Y., U.S.A.). Cetavlon (cetyltrimethylammonium bromide) was from British Drug Houses Ltd. (Poole, Dorset).

Methods

Thin-layer chromatography. The phosphatides were separated on chromatoplates prepared according to Mangold (1962), with the solvent system chloroform–methanol–water (65:25:4, by vol.) (Wagner, Hörhammer & Wolff, 1961). Ceramides were chromatographed with chloroform–methanol–acetic acid (95:1:5, by vol.). Phosphorylcholine was identified on thin-layer chromatography by the solvent system described by Skidmore & Entenman (1962). The spots were made visible by iodine vapour or by sulphosalicylic acid reagent. Protein determinations were carried out according to Lowry, Rosebrough, Farr & Randall (1951), with bovine serum albumin as standard. Phosphorus was estimated by a slight modification to the methods of Fiske & Subbarow (1925) and Bartlett (1959) and nitrogen was determined by a slight modification to the method of Lang (1958).

Tissue fractionations. Rat livers were homogenized in 4 vol. of 0·25 M-sucrose in 1 mM-tris–HCl buffer, pH 7·4. These homogenates were subjected to differential centrifugation, according to the scheme suggested by de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955). The centrifugations were performed with a Servall centrifuge, rotor no. SS–34, at 0–4°. The nuclear fraction (N) was obtained by centrifugation of the homogenate at 600 *g* for 10 min. The heavy-mitochondrial fraction (M) was obtained by centrifuging the nuclear supernatant for 10 min. at 3500 *g*. The light-mitochondrial fraction (L) was a sediment of the heavy-mitochondrial supernatant subjected to a centrifugal force of 18000 *g* for 20 min. The rest, namely the microsomal fraction and cell sap (post-mitochondrial fraction), were not separated further and were designated (S). All sediments were washed once with the suspending medium (0·25 M-sucrose in 1 mM-tris–HCl buffer, pH 7·4), centrifuged and resuspended in the original volume.

Purification of enzyme. The rat-liver homogenates in 0·25 M-sucrose containing 1 mM-tris–HCl buffer, pH 7·4, were centrifuged at 600 *g* for 10 min. to remove the nuclei and cell debris, and the particles that sedimented between 600 *g* and 18000 *g* were used. All operations were carried out at 0–4° unless otherwise stated. The particle suspension was subjected to ultrasonic treatment for several minutes and subsequently to repeated freezing and thawing (about 10 times), between −70° (acetone–solid CO_2) and 50°. This was followed by centrifugation for 90 min. at 105 000 *g* in a Spinco model L preparative ultracentrifuge. The precipitate was discarded and the clear supernatant (supernatant 1) was acidified with dilute acetic acid to bring it to pH 5·2–5·3. After centrifugation at 10 000 *g* for 20 min. the supernatant was discarded, and the sediment (precipitate pH 5·3) was resolubilized by adding 0·25 M-sucrose containing 0·1 M-tris–HCl buffer, pH 7·4, and 0·08% of sodium cholate. All the insoluble material was removed by centrifugation at 105 000 *g* for 90 min. (supernatant 2). The clear yellowish supernatant was then subjected to fractionation with $(NH_4)_2SO_4$ at pH 7·5–7·8. Ammonium sulphate was added as the solid; the fraction precipitating between 15 and 40% (w/v) contained most of the enzyme.

Assays of enzyme activity. Total acid-phosphatase (EC 3.1.3.2) activity was assayed by the method of Wattiaux & de Duve (1956), with some modification in the presence of 0·1% of Triton X-100 as releasing agent. The buffer was 0·05 M-pyridine–HCl, pH 5·5, and the substrate was 0·01 M-*p*-nitrophenyl phosphate. The reaction was stopped after 10 or 20 min. by the addition of 5% trichloroacetic acid, the mixture was centrifuged and a sample taken to measure the production of *p*-nitrophenol at 400 mμ after the addition of 0·5 N-NaOH. The activity is expressed as μmoles of *p*-nitrophenol formed/min.

Sphingomyelinase. In the earlier experiments this enzyme was assayed by determining the decrease in sphingomyelin (Heller & Shapiro, 1963). In later experiments the formation of water-soluble phosphorus compounds was followed. The reaction mixture of 0·5 ml. or 1 ml. final volume contained 1·5–3·2 μg.atoms of sphingomyelin phosphorus, 0·05–0·075 M-buffer (pyridine–HCl or acetate–acetic acid), pH 5·5, 2 mg. of Triton X-100 and the enzyme (the amount of protein used did not exceed 1 mg./ml.). Incubations were carried out at 37° in glass-stoppered tubes with shaking for 90–180 min. The reaction was terminated by adding 6 ml. of chloroform–methanol (2:1, v/v). Potassium chloride solution was added to form the two-phase system of Folch *et al.* (1957). The amount of phosphorus in the upper phase was measured. Units of activity were defined as the amount of phosphorylcholine (μmoles/min.) formed under the conditions specified. The specific activity of the enzyme was defined as units/mg. of protein.

All the estimations of enzymic activity were performed under optimum conditions, giving zero-order kinetics. This was always checked by carrying out the assays with two enzyme concentrations and frequently with two incubation periods.

RESULTS

Nature of the enzymic reaction. In preliminary observations (Heller & Shapiro, 1963) it was found that rat-liver homogenates can hydrolyse sphingomyelin, since the amount of phosphatide resistant to both mild alkali and acid treatments decreases. Examination of the water-soluble and lipid products of reaction showed clearly that the reaction is a hydrolytic cleavage of sphingomyelin:

Sphingomyelin + water ⟶ ceramide + phosphorylcholine

Intracellular distribution of the enzyme. It was revealed that the sphingomyelinase activity is associated with particles sedimenting between 600 and 18000 *g*, which contain both heavy- and light-mitochondrial fractions. No activity could be detected in the microsomal fraction or the cell sap.

To correlate the sphingomyelinase with the subcellular fraction, acid phosphatase, which is known to be located in the lysosomes (de Duve, 1959), was chosen as a reference enzyme (Table 1). It seems that most of the sphingomyelinase activity accompanies the acid phosphatase. Further evidence for the localization of sphingomyelinase in the lysosomes was obtained by keeping fresh rat livers frozen for several days; on thawing and centrifuging homogenates prepared therefrom at 105000 *g* for 90min., no activity was sedimented, but all the enzyme was found in the soluble cell sap. In addition it is known that Triton X-100 releases the lysosomal hydrolases into solution and a 0·5% solution of this non-ionic detergent was found to liberate sphingomyelinase.

Purification. The particles obtained by centrifuging the homogenate between 600 and 18000 *g* and which are known to contain mitochondria and lysosomes served as the source of enzyme for purification. Although Triton X-100 released the enzyme from these particles, it also solubilized the bulk of the other mitochondrial and lysosomal proteins. Another disadvantage in using this detergent was the difficulty of removing it for further purification. de Duve (1959) has suggested several agents and treatments for disruption of the lysosomal membrane to release the hydrolases. Accordingly we tried to treat the particles ultrasonically for 3–20min., but found that after this treatment and high-speed centrifugation the separation of the enzyme from the total protein in the soluble and insoluble fractions was inadequate for further purification. However, when ultrasonic treatment was followed by repeated freezing and thawing, the supernatant obtained after high-speed centrifugation contained most of the enzyme with only 30% of the total protein. The purification procedure is given in the Experimental section; Table 2 summarizes the purification pattern, indicating a 21-fold purification. The calculated enzyme activity of fresh liver is 0·31 unit/g.

pH-*dependence.* The optimum activity was between pH 5 and pH 5·5 with different buffer systems. Pyridine–hydrochloric acid, phthalate–sodium hydroxide and acetic acid buffers at 0·05–0·1 M gave the best results, whereas in citrate and maleate systems lower values were obtained. Fig. 1 shows the pH curves as measured with two different buffer systems.

Table 1. *Distribution pattern of sphingomyelinase and acid phosphatase*

The subcellular fractionation pattern is described in the Methods section. 'Relative specific activity' is the ratio between the percentage of total activity and the percentage of total protein.

			Acid phosphatase		Sphingomyelinase	
Fraction	Volume (ml.)	Protein (%)	Activity (%)	Rel. sp. activity	Activity (%)	Rel. sp. activity
Homogenate	24	100	100	—	100	—
Nuclear fraction	29	34	22	0·65	19	0·56
Heavy mitochondria	24·5	9	7·8	0·87	19	2·1
Light mitochondria	26·5	15	40·5	2·7	60	4·0
Post-mitochondrial fraction	120	41	30	0·73	0	0

Table 2. *Purification of sphingomyelinase*

Homogenate was obtained from 200g. of rat liver in a final volume of 1l. 'Particles': heavy and light mitochondria. 'Supernatant 1': obtained after high-speed centrifugation of 'particles' treated ultrasonically and by freezing and thawing. 'Precipitate (pH 5·3)': obtained from 'supernatant 1'. Supernatant 2: the supernatant obtained after neutralizing 'precipitate (pH 5·3)' to pH 7·5 and high-speed centrifugation. 'Ammonium sulphate': the precipitate obtained from 'supernatant 2' by ammonium sulphate at 15–40% (w/v) concn. Incubation time, 120min.

Fraction	Protein (g.)	Activity (units)	$10^3 \times$ Sp. activity	Yield (%)	Purification
Homogenate	51	68·0	1·33	100	1
Particles	10·2	48·5	4·75	71	3·55
Supernatant 1	3·6	28·2	7·82	41·5	5·9
Precipitate (pH 5·3)	1·8	21·5	11·9	31·5	8·9
Supernatant 2	1·31	20·8	15·9	30·5	12
Ammonium sulphate	0·36	10·3	28·5	15	21·6

Kinetics. The hydrolysis of sphingomyelin by the enzyme was linear with time for at least 4hr. Proportionality was also found with enzyme concentration (Fig. 2). The dependence of the velocity on substrate concentration indicated an apparent K_m 0·5–0·9 mM (Fig. 3).

Activation and inhibition. Both the crude homogenate and the particles were capable of hydrolysing sphingomyelin without any other additions. The partially purified preparations, however, required some surface-active agent such as Triton X-100 or Cetavlon. Emulsification of

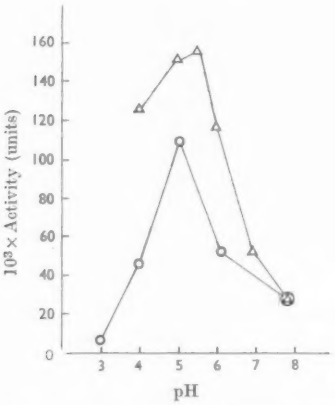

Fig. 1. pH–activity curve of sphingomyelinase. ○, Acetate–acetic acid buffer, 0·05 M; △, pyridine–HCl buffer, 0·05 M.

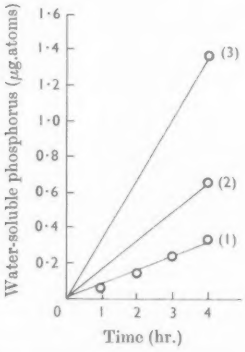

Fig. 2. Kinetics and enzyme concentration-dependence of sphingomyelinase. This was performed with the 'ammonium sulphate fraction' (see Table 1). Enzymic protein: (1) 0·21 mg.; (2) 0·42 mg.; (3) 0·84 mg.

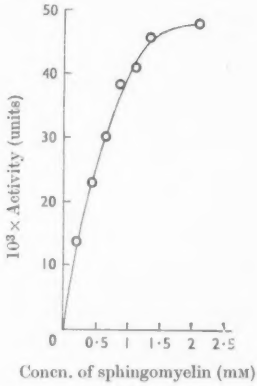

Fig. 3. Dependence of rate on substrate concentration. All components of the reaction mixture were constant, but sphingomyelin concentration was varied from 0·2 mM to 2 mM as shown. The experiment was performed with 'supernatant 1' fraction (0·45 mg. of protein). Incubation time, 90 min.

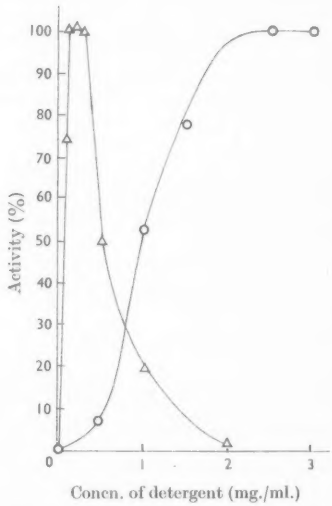

Fig. 4. Effect of Triton X-100 (○) and Cetavlon (△) on sphingomyelinase activity. Both detergents were added to the reaction mixture, as aqueous solutions in the concentrations (mg./ml.) indicated, at pH 5·5. The experiment was performed with 'supernatant 1' fraction; 100% for Triton X-100 effect was 0·35 μg.atom of 'water-soluble phosphorus' formed; 100% for Cetavlon effect was 0·31 μg.atom of 'water-soluble phosphorus' formed. Incubation time, 120 min.

sphingomyelin by ultrasonic treatment for 8min. was insufficient to initiate enzymic hydrolysis. The activating effect of Triton X-100 reached a plateau at approx. 2mg./ml. Cetavlon gave maximal results at a concentration of 0·2mg./ml., higher concentrations causing inhibition (Fig. 4). When Triton X-100 was added to inhibitory amounts of Cetavlon, the inhibition was not counteracted. The surface-active agent may be required for both solubilization of the enzyme, which is precipitated at the pH of the reaction, and for proper dispersion of the substrate. The addition of diethyl ether or Ca^{2+} ions, either alone or in combination, or the addition of chelating agents such as EDTA, citrate or maleate, was essentially without effect. Oleic acid (1mM) or sodium chloride (up to 0·5M) did not influence the rate of hydrolysis in the presence of Triton X-100. In the absence of the detergent no activity could be detected under any of the above conditions.

Stability. The enzyme is heat-labile, being inactivated after 1min. in boiling water or 10min. at 65° in the absence of the substrate.

The enzyme preparations, at various stages of purification, were relatively stable when kept frozen at −18°. A 30–35% loss of activity during a period of about 3 months was observed when the enzyme solutions were repeatedly withdrawn from the freezer for assay, thawed and refrozen. This relative stability was greatly dependent on the presence of some preserving agent. Sucrose (0·25M), glycerol (20%) or ammonium sulphate (33% saturation) were satisfactory agents, sucrose being the one generally used. In solutions containing buffer only, more than 80% loss in activity was observed within a day of storage at −18°.

Specificity. The phospholipases C from microbial sources are relatively non-specific, hydrolysing most of the phosphatides (Kates, 1960; Matsumoto, 1961). The rat-liver enzyme has a much greater specificity. Marked differences in rates of hydrolysis were found with different sphingomyelin isomers. The naturally occurring sphingomyelin (which has been shown to be D-*erythro-trans*-2-*N*-acyl-1-*O*-phosphorylcholinesphingosine; Shapiro & Flowers, 1962), as well as synthetic D- or DL-*erythro* isomer, were hydrolysed at approximately the same rate. Synthetic sphingomyelins containing dihydrosphingosine were hydrolysed by the enzyme to an extent almost equal to those containing sphingosine. Much lower activity, however, was found with the *threo* isomer of the dihydrosphingomyelin tested (Table 3). The following phospho compounds tested were hydrolysed at a much slower rate than sphingomyelin: ovolecithin, synthetic lecithin, phosphorylcholine.

Phosphatidic acid, however, was split almost at the same rate as sphingomyelin by the crude particles. These particles contain a specific

Table 3. *Action of sphingomyelinase on sphingomyelins from various sources*

Expt. 1 was performed with 'particles' (0·96mg. of protein in the reaction mixture). Expt. 2 was performed with 'supernatant 1' (0·75mg. of protein in the reaction mixture) (see Table 1).

Expt.	Substrate	Source	Substrate added (μmoles)	Product formed (μmole)
1	Sphingomyelin	Rat liver	1·79	0·665
		Red blood cells	1·58	0·560
		Sheep liver	1·64	0·495
		Cattle liver	1·56	0·485
		Cattle brain	1·40	0·705
	D-*erythro*-*N*-Palmitoyl-sphingomyelin	Synthetic	0·72	0·315
			1·44	0·515
			2·16	0·681
			2·88	0·762
	DL-*threo*-*N*-Palmitoyl-dihydrosphingomyelin	Synthetic	1·44	0·247
			2·16	0·167
			2·88	0·180
			3·60	0·145
			4·32	0·187
2	Sphingomyelin	Rat liver	1·00	0·332
	D-*erythro*-*N*-Palmitoyl-sphingomyelin	Synthetic	1·00	0·322
	DL-*erythro*-*N*-Palmitoyl-dihydrosphingomyelin	Synthetic	1·28	0·269
	DL-*threo*-*N*-Palmitoyl-dihydrosphingomyelin	Synthetic	1·00	0·02
			2·00	0·08

phosphatidic acid phosphatase, as shown by Wilgram & Kennedy (1963), but this activity was lost during purification. It could no longer be detected in the 'ammonium sulphate fraction', even when tested at pH 7·5, known to be at the optimum of this enzyme (Table 4). The acid-phosphomonoesterase activity toward *p*-nitrophenyl phosphate is, however, carried along during the purification steps.

Product inhibition. To test whether a product inhibition occurs, both ceramide and phosphorylcholine were added, one at a time or in combination, with the concentrations varied from 0·5 to 1·6 mM. Only 23% inhibition was obtained.

Stoicheiometry of the reaction. A reaction mixture (containing purified enzyme) was incubated and the reaction terminated as described above; the upper methanol–water phase was analysed for total phosphorus and for inorganic phosphorus. Since no inorganic phosphorus was found, the total phosphorus represented phosphorylcholine. The lower phase was analysed for total phosphorus.

Another sample of the lower phase was evaporated to dryness under nitrogen and the residue was extracted with propan-2-ol–heptane–sulphuric acid according to Dole (1956). The heptane layer contained all the ceramide (Roitman & Gatt, 1963). However, some sphingomyelin was also carried into

Table 4. *Action of sphingomyelinase on phosphorus-containing substrates*

Expts. 3 and 4 were performed with 'particles' (0·9 mg. of protein and 0·31 mg. of protein respectively). Expt. 5 was performed with 'supernatant 1' (0·6 mg. of protein). Expts. 6 and 7 were performed with 'ammonium sulphate fraction' (0·34 mg. of protein and 0·15 mg. of protein respectively). All the reactions were carried out at the pH optimum of sphingomyelinase (5–5·5), except the experiments marked with asterisks, which were performed also at pH 7·5 for comparison. (Note: the hydrolysis of phosphorylcholine and phosphatidic acid was followed by measuring production of inorganic phosphorus.)

Expt.	Substrate	Source	Substrate added (μmoles)	Product formed (μmole)
3	Sphingomyelin	Rat liver	1·56	0·4
	Lecithin	Egg	1·63	0·023
	Lecithin (dipalmitoyl)	Synthetic	1·52	0·032
4	Sphingomyelin	Rat liver	1·05	0·160
	Phosphatidic acid	Egg lecithin	1·15	0·143
	Phosphorylcholine	Synthetic	1·05	0·038
5	Sphingomyelin	Cattle brain	1·68	0·335
	Sphingomyelin	Cattle brain	1·68	0·118*
	Phosphatidic acid	Egg lecithin	1·4	0·091
	Phosphatidic acid	Egg lecithin	1·4	0·121*
6	Sphingomyelin	Cattle brain	1·56	0·36
	Lecithin	Egg	1·0	0
			2·0	0·037
7	Sphingomyelin	Cattle brain	1·68	0·183
	Sphingomyelin	Cattle brain	1·68	0·038*
	Phosphatidic acid	Egg lecithin	1·4	0
	Phosphatidic acid	Egg lecithin	1·4	0*

Table 5. *Stoicheiometry of the reaction*

Experiments were performed with the purified enzyme at the stage of 'ammonium sulphate fraction'. Results are expressed in μg.atoms or μmoles. Sphingomyelin was assayed as 'chloroform-soluble phosphorus', phosphorylcholine as 'water-soluble phosphorus' (Folch *et al.* 1957), ceramide as 'heptane-soluble nitrogen' (Dole, 1956).

Expt.	Time (min.)	Sphingomyelin	Ceramide	Phosphoryl-choline	P_i
1	0	1·56	0	0	—
	150	0·72	1·12	1·01	—
2	0	5·32	0	0	—
	150	2·56	2·50	2·0	—
3	0	6·3	0	0	—
	150	3·76	3·46	2·54	—
4	0	6·73	0	0	0
	150	4·70	1·9	2·03	0

this phase, as shown by phosphorus analysis of this phase and by thin-layer chromatography. Therefore the heptane layer was washed with 'Dole's lower phase solvents' and the interfering sphingomyelin could be removed almost completely (phosphorus estimations revealed removal of more than 95% of the accompanying sphingomyelin). The amount of ceramide was estimated by determining total nitrogen in the washed heptane phase. Table 5 shows four analyses done by this procedure.

DISCUSSION

The enzymic breakdown of sphingomyelin by rat-liver preparations, demonstrated in this paper, is similar to the cleavage brought about by phospholipase C from various bacterial sources, yielding phosphorylcholine and ceramide. The animal phospholipase C differs, however, from the bacterial enzyme in its substrate specificity, showing very little activity towards lecithin. Further, the configuration of the sphingosine moiety influences the enzymic activity considerably.

No cleavage of the fatty acid from sphingomyelin was found, even with crude liver preparations. It seems likely therefore that the sequence of degradation of sphingomyelin is to form ceramide, which may then be hydrolysed to sphingosine and fatty acid by an enzyme described by Gatt (1963) in rat tissues.

Part of this work is included in a Ph.D. Thesis submitted by M. H. to the Senate of the Hebrew University, Jerusalem.

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The Comparative Metabolism of 2,6-Dichlorothiobenzamide (Prefix) and 2,6-Dichlorobenzonitrile in the Dog and Rat

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1. A single oral dose of either [^{14}C]Prefix or 2,6-dichlorobenzo[^{14}C]nitrile to rats is almost entirely eliminated in 4 days: 84·8–100·5% of ^{14}C from [^{14}C]Prefix is excreted, 67·3–79·7% in the urine, and 85·8–97·2% of ^{14}C from 2,6-dichlorobenzo-[^{14}C]nitrile is excreted, 72·3–80·7% in the urine. Only $0{\cdot}37 \pm 0{\cdot}03$% of the dose of [^{14}C]Prefix and $0{\cdot}25 \pm 0{\cdot}03$% of the dose of 2,6-dichlorobenzo[^{14}C]nitrile are present in the carcass plus viscera after removal of the gut. Rats do not show sex differences in the pattern of elimination of the respective metabolites of the two herbicides. The rates of elimination of ^{14}C from the two compounds in the 24 hr. and 48 hr. urines are not significantly different ($P > 0{\cdot}05$) from one another. 2. After oral administration to dogs, 85·9–106·1% of ^{14}C from [^{14}C]Prefix is excreted, 66·6–80·9% in the urine, and 86·8–92·5% of ^{14}C from 2,6-dichlorobenzo[^{14}C]nitrile is excreted, 60·0–70·1% in the urine. Dogs do not show sex differences in the pattern of eliminating the metabolites of either Prefix or 2,6-dichlorobenzonitrile. 3. Dogs and rats do not show species differences in the patterns of elimination of the two herbicides. 4. Prefix and 2,6-dichlorobenzonitrile are completely metabolized; unchanged Prefix and 2,6-dichlorobenzonitrile are absent from the urine and faeces, and from the carcasses when elimination is complete. In the hydrolysed urine of rats dosed with either [^{14}C]Prefix or 2,6-dichlorobenzo[^{14}C]nitrile, 2,6-dichloro-3-hydroxybenzonitrile accounts for approx. 42% of the ^{14}C, a further 10–11% is accounted for by 2,6-dichlorobenzamide, 2,6-dichlorobenzoic acid, 2,6-dichloro-3- and -4-hydroxybenzoic acid and 2,6-dichloro-4-hydroxybenzonitrile collectively, and 25–30% by six polar constituents, of which two are sulphur-containing amino acids. 5. In the unhydrolysed urines of rats dosed with either [^{14}C]Prefix or 2,6-dichlorobenzo[^{14}C]nitrile, there are present free 2,6-dichloro-3- and -4-hydroxybenzonitrile, their glucuronide conjugates, ester glucuronides of the principal aromatic acids that are present in the hydrolysed urines, and two sulphur-containing metabolites analogous to mercapturic acids or premercapturic acids. 6. Prefix is thus extensively transformed into 2,6-dichlorobenzonitrile: $R \cdot CS \cdot NH_2 \rightarrow R \cdot CN + H_2S$, where $R = C_6H_3Cl_2$. However, the competitive reaction: $R \cdot CS \cdot NH_2 + H_2O \rightarrow R \cdot CO \cdot NH_2 + H_2S$ takes place to a very limited extent.

2,6-Dichlorothiobenzamide (Prefix) (Yates, 1961) and 2,6-dichlorobenzonitrile (Belgian Patent, 1957) are promising herbicides with interesting biological properties (Barnsley, 1960; Koopman & Daams, 1960). Prefix is useful as a selective herbicide in transplanted rice. Agricultural application makes an understanding of the mammalian metabolism of the two herbicides desirable.

MATERIALS

2,6-Dichlorobenzonitrile. This was prepared by dehydration in boiling acetic anhydride of *anti*-2,6-dichlorobenzaldoxime (Koopman, 1961), which was itself prepared by reaction of 2,6-dichlorobenzaldehyde (Fierz-David & Blangey, 1952; Reich, 1917) with hydroxylamine monosulphonate (Koopman, 1961). 2,6-Dichlorobenzonitrile had m.p. 145° (Koopman, 1961, gives 144–145°).

2,6-Dichlorothiobenzamide (Prefix). This was prepared by the addition of H_2S to a solution of 2,6-dichlorobenzonitrile in dimethylformamide at 60°, according to the method of Yates (1961). Prefix had m.p. 152° (Yates, 1961, gives m.p. 152°).

Purity of the herbicides. The two herbicides, which had been crystallized from benzene and ethanol to constant melting point, migrated as single substances on thin-layer plates of Kieselgel G run with benzene; phenoxyethanol–$AgNO_3$ spray (Mitchell, 1958) was used for locating the substance on the thin-layer plate. Infrared measurement

was used as a further criterion of purity. The purity of each compound exceeded 99·9%.

The unlabelled herbicides were used for dosing rats, in which the cysteine–cystine pools had been labelled with ^{35}S (see the Methods section). Unlabelled herbicides were also used as reference compounds to establish the presence or absence of unchanged ^{14}C-labelled herbicides in the urine and faeces of treated animals (see the Results section).

2,6-Dichlorobenzoic acid. This compound, m.p. 146–147°, was prepared from 2,6-dichlorobenzaldehyde (Fierz-David & Blangey, 1952; Reich, 1917) by oxidation with $KMnO_4$ in acetone solution (Davies, 1921).

2,6-Dichlorobenzamide. This compound, m.p. 202°, was prepared by treatment of 2,6-dichlorobenzonitrile with alkaline H_2O_2 (Reich, Salzmann & Kawa, 1917).

2,6-Dichloro-3-hydroxybenzonitrile. This was prepared from 2,6-dichloro-3-hydroxybenzaldehyde (Hodgson & Beard, 1926) as starting material. When the corresponding aldoxime (5·6g.) (Hodgson & Beard, 1926) was refluxed in 20ml. of acetic anhydride for 2hr. and poured into water, 2,6-dichloro-3-acetoxybenzonitrile was obtained as an oil, which crystallized (5·1g., m.p. 106°). Hydrolysis, by refluxing for 1hr. with 3N-KOH under N_2, and acidification yielded 2,6-dichloro-3-hydroxybenzonitrile (4·1g., m.p. 196–198°) (Yates, 1962).

2,6-Dichloro-3-hydroxybenzoic acid. This was prepared by oxidation of 2,6-dichloro-3-hydroxybenzaldehyde (Hodgson & Beard, 1926) with freshly prepared Ag_2O according to the method of Pearl (1950). 2,6-Dichloro-3-hydroxybenzoic acid crystallized from water, forming prisms of the monohydrate, m.p. 126° (Mazzara & Bertozzi, 1900, give m.p. 123–124° for the monohydrate). *Methyl 2,6-dichloro-3-methoxybenzoate.* This compound, m.p. 57° (Mazzara & Bertozzi, 1900), was used as a reference compound in the gas–liquid chromatography (see the Methods section and Table 7). [The methyl ester and the two *O*-methyl ethers, which were also used in gas–liquid chromatography (see Table 7), were oils that failed to crystallize, and are not therefore described in the Materials section.]

2,6-Dichloro-4-hydroxybenzonitrile. This was synthesized from *p*-nitrosophenol as starting material. Reaction of an ethanolic solution of *p*-nitrosophenol with HCl yielded 2,6-dichloro-4-ethoxyaniline (Bargellini, 1929), which was converted into 2,6-dichloro-4-hydroxybenzonitrile by successive diazotization, Sandmeyer reaction and de-ethylation. This reaction sequence gave a better yield of final product from the same intermediate, 2,6-dichloro-4-ethoxyaniline, than the alternative sequence, de-ethylation, diazotization and Sandmeyer reaction, used by Strating & Backer (1943). 2,6-Dichloro-4-hydroxybenzonitrile crystallized from toluene, forming needles, m.p. 229–230° (Strating & Backer, 1943, give m.p. 228–230°).

2,6-Dichloro-3-hydroxythiobenzamide. This compound, m.p. 197–198°, was supplied by Dr J. Yates.

Bovine liver β-glucuronidase. This enzyme (1g., 560000 units) (Fishman, Springer & Brunetti, 1948) was obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A., and was found to be free from hippuricase and sulphuric ester hydrolase activities.

Reagents and solvents. All reagents and solvents were of A.R. grade.

Radioactive chemicals. 2,6-Dichlorobenzo[^{14}C]nitrile was synthesized by reaction of 2,6-dichloroiodobenzene (Körner & Contardi, 1913) with $Cu^{14}CN$ and purified by column chromatography on silicic acid. 2,6-Dichlorobenzo[^{14}C]-nitrile (sp. activity 9·1 μc/mg.) was eluted with methylene dichloride.

2,6-Dichlorothiobenz[^{14}C]amide ([^{14}C]Prefix) was prepared by addition of H_2S to 2,6-dichlorobenzo[^{14}C]nitrile (sp. activity 3·2 μc/mg.) and purified by column chromatography on silicic acid. [^{14}C]Prefix (sp. activity 3·2 μc/mg.) was eluted with methylene dichloride.

The two ^{14}C-labelled herbicides behaved identically with authentic specimens of the unlabelled compounds on thin-layer plates of Kieselgel G run with benzene; radioautography did not reveal radioactive impurities. The radiochemical purity of both compounds exceeded 99·9%.

L-[^{35}S]Cystine (sp. activity 424 μc/mg.) was obtained from The Radiochemical Centre, Amersham, Bucks.

METHODS

Experiments with animals. Adult rats (approx. 2 months old, 150–200g. body wt.) were used (Porton strain maintained as a specific pathogen-free colony in this Laboratory). For the metabolism experiments the animals were kept in all-glass metabolism cages under the conditions described by Wright, Akintonwa, Crowne & Hathway (1965). In the first experiment a solution of [^{14}C]Prefix (1·8mg., 3·2 μc/mg.) in 2ml. of aq. 5% (v/v) ethanol was administered to male and female rats by stomach tube, and in the second experiment 0·80mg. (males) or 1·3mg. (females) of 2,6-dichlorobenzo[^{14}C]nitrile (9·1 μc/mg.) in 2ml. of arachis oil was administered to rats. Unrestricted food and water were supplied, the urine and faeces were collected daily and stored at −29° and the expired gases were monitored for $^{14}CO_2$. In these experiments the rats were killed after 4 days and the alimentary canal was removed. The carcass plus viscera, the alimentary canal and the skin plus hair were stored separately at −29°.

In another experiment 1·5ml. of an aq. 2% (w/v) $NaHCO_3$ solution containing 13 μc of L-[^{35}S]cystine/ml. was administered intraperitoneally to four rats of each sex on 5 consecutive days. On the fourth and fifth days two rats of each sex were each dosed by stomach tube with 1ml. of a solution of Prefix (5mg.) in arachis oil and two rats of each sex were each dosed by stomach tube with 1ml. of a solution of 2,6-dichlorobenzonitrile (5mg.) in arachis oil. Unrestricted food and water were supplied and the urine was collected on the fourth and fifth days and stored at −29°. This experiment is based on work of Gutmann & Wood (1950) and Marsden & Young (1958).

Adult hounds (7–15kg. body wt.) were used (beagles maintained for 2–3 years as a closed colony in this Laboratory). For the metabolism experiments the animals were kept in stainless-steel metabolism cages under the conditions described by Wright *et al.* (1965). In one experiment each dog swallowed a gelatin capsule containing a solution of 7·90mg. of [^{14}C]Prefix (2·8 μc/mg.) in ethanol (1ml.), and in another experiment each dog swallowed a gelatin capsule containing a solution of 0·92mg. of 2,6-dichlorobenzo[^{14}C]-nitrile (8·52 μc/mg.) in 1ml. of arachis oil. Unrestricted food and water were supplied and the urine and faeces were collected separately at daily intervals.

Fractionation of urines from treated animals. Solvent extracts of the pooled urines from 12 rats were made by continuous extraction at pH 1·5–2·0 in a Schacherl apparatus with ether and ethyl acetate successively. Evaporations

were carried out in N_2 under reduced pressure at below 35°.

Hydrolysis of urines from treated animals and fractionation of the hydrolysed urines. Preliminary investigations showed that hydrolysis of the ^{14}C-labelled metabolites in the urines of treated animals gave the same chromatographic and electrophoretic patterns of ether-soluble ^{14}C-labelled constituents irrespective of whether the hydrolysing agent was β-glucuronidase or 6N-HCl (3hr. at 110°). The alternative hydrolytic procedures did, however, yield some different ^{14}C-labelled constituents in the residual aqueous phases after ether extraction. Thus the ^{14}C-labelled sulphur-containing metabolites (see below) were transformed by acid hydrolysis into ^{14}C-labelled sulphur-containing amino acids, whereas they were unaffected by enzymic hydrolysis. For the purpose of identifying ^{14}C-labelled constituents in urine hydrolysates, the pooled urines of 12 treated animals were hydrolysed with acid. The hydrolysed urines were exhaustively extracted by continuous liquid–liquid extraction (Schacherl apparatus) with ether and ethyl acetate successively, and the sum of the separate amounts of ^{14}C in the solvent extracts and residual aqueous phase accounted quantitatively for the original label in the unhydrolysed urine. Further, when the individual compounds in the solvent extracts and residual aqueous phase were separated on paper chromatograms and electrophoretograms (for details see below), which in turn were scanned and summed, an approximate balance of radioactivity was obtained. This showed that acid hydrolysis did not cause destruction of hydrolytic products with consequent loss of radioactivity as ^{14}C, $^{14}CO_2$ or ^{14}C-labelled precipitates. This work was reproducible; other batches of pooled urines from 12 rats dosed with the ^{14}C-labelled herbicides, when hydrolysed with acid in this way, also gave a consistent balance of ^{14}C and identical proportions of the same ^{14}C-labelled constituents.

Samples (5mg.) of each of the ^{14}C-labelled constituents that were subsequently identified in the ether phase (2,6-dichlorobenzamide, 2,6-dichloro-3- and -4-hydroxybenzonitrile, 2,6-dichlorobenzoic acid and 2,6-dichloro-3-hydroxybenzoic acid) and 2,6-dichloro-3-hydroxythiobenzamide were heated separately with 6N-HCl (5ml.) for 3hr. at 110°. Paper chromatograms of the ether extracts of the separate reaction mixtures were run in butan-1-ol saturated with 2N-NH_3, and these were identical in every respect with the chromatograms of the initial authentic compounds. In each case, unchanged starting material was recovered quantitatively from the reaction mixtures. These experiments demonstrate the great stability of the hindered amido, cyano and thioamido groups in these molecules and the lack of reactivity of the chloro substituents in 2,6-positions. Since these constituents were stable to acid and since, with the exception of 2,6-dichloro-3-hydroxythiobenzamide, they were formed by similar acid hydrolysis of the urines of animals dosed with the two ^{14}C-labelled herbicides, it follows that the reactions by which they were produced from the (conjugated) metabolites in urine were probably straightforward hydrolytic reactions.

Chromatography on a silicic acid column. Mallinckrodt (100 mesh) silicic acid was washed and reactivated as described by Wright *et al.* (1965). Ether-soluble ^{14}C-labelled metabolites were successively eluted with solvents of increasing polarity; a typical fractionation is set out in Table 7.

Paper chromatography. Unconjugated ^{14}C-labelled metabolites of [^{14}C]Prefix and 2,6-dichlorobenzo[^{14}C]nitrile were separated by descending chromatography on Whatman no. 1 filter-paper sheets at 29°, and Table 8 shows a typical separation of the ether-soluble ^{14}C-labelled metabolites. Paper chromatography in the same solvent system was used for separating the more polar unconjugated ^{14}C-labelled metabolites and the ^{14}C-labelled conjugates in the unhydrolysed urines. All ^{14}C- or ^{35}S-labelled constituents were detected and identified on paper by radioactive scanning with a Nuclear–Chicago Actigraph II, equipped with a gas-flow cell utilizing a helium–*n*-butane (98·5:1·5, v/v) mixture. Phenoxyethanol–$AgNO_3$ (Mitchell, 1958) was used as location reagent, and the fluorescence of some unconjugated metabolites in ultraviolet light served as another means of location.

Thin-layer chromatography. This was carried out on Kieselgel G (E. Merck A.-G., Darmstadt, Germany) as described by Wright *et al.* (1965). The ^{14}C-labelled metabolites were detected and identified on thin plates by radioactive scanning with a Desaga radiochromatogram scanner. Thin-layer chromatography was useful for separating ^{14}C-labelled aromatic acids and [^{14}C]phenols; acetone–*n*-hexane (1:1, v/v) was used as solvent system. The purity of the two ^{14}C-labelled herbicides was checked by thin-layer chromatography, benzene being the preferred solvent for development.

Gas–liquid chromatography. Chromatographs, each equipped with an electron-capture detector, were used for comparing retention times of ^{14}C-labelled metabolites with those of reference compounds. For the methyl esters, columns of working length 4ft. were filled with Diatopore S that had been coated with 3·8% of SE30 silicone oil as stationary phase. The running conditions were: temperature, 120°; inlet pressure, 20lb./in.^{2}; flow rate for N_2, not known. For the *O*-methoxy and neutral constituents, columns of working length 2ft. were filled with untreated Celite (100–120 mesh) that had been coated with 5% of phenyl diethanolamine succinate as stationary phase. The running conditions were: temperature, 188°; flow rate for N_2, 50ml./min.

Electrolyte solutions. Formic acid–acetic acid buffer, pH 2·0, was prepared by diluting a mixture of 98–100% formic acid (78ml.) and 99·6% acetic acid (148ml.) with water to 2·5l. Acetate–acetic acid buffer, pH 5·2, was made by mixing 0·2N-acetic acid (200ml.) and 0·2M-sodium acetate (800ml.). Veronal–HCl buffer, pH 9·0, was prepared by mixing 936ml. of 0·1M-veronal and 64ml. of 0·1N-HCl.

Low-voltage electrophoresis. After electrophoresis on strips of Whatman no. 1 filter paper in veronal–HCl buffer, pH 9·0, at 12v/cm., [^{14}C]phenols and ^{14}C-labelled aromatic acids had migrated towards the anode; under these conditions, ^{14}C-labelled phenolic acids were separated from ^{14}C-labelled aromatic acids. Electrophoresis in acetate buffer, pH 5·2, at 12v/cm. caused ^{14}C-labelled aromatic acids to migrate towards the anode. After electrophoresis the paper strips were infrared-heated in an air stream for 10min. to remove most of the liquid and were subsequently dried at 80° for 15min. Zones of radioactivity were scanned with the Nuclear–Chicago Actigraph 4π system.

High-voltage electrophoresis. Electrophoresis on strips of Whatman no. 1 filter paper in formic acid–acetic acid buffer, pH 2·0, in 1hr. at 120v/cm. (Hathway, Mallinson & Akintonwa, 1965) caused movement of a ^{14}C- or ^{35}S-labelled

polar constituent towards the cathode with one-half of the speed of glycine, whereas low-voltage electrophoresis in veronal–HCl buffer, pH 9·0, caused the same ^{14}C- or ^{35}S-labelled polar constituent to migrate towards the anode. The dried electrophoretograms were scanned for radioactive zones.

Measurement of radioactivity. A Tri-Carb spectrometer (Packard Instrument Co. Inc., La Grange, Ill., U.S.A.) was used for the measurement of ^{14}C by liquid-scintillation counting. Measurements of radioactivity in urine, faeces, gut and contents, carcass plus viscera and skin plus hair and of the $^{14}CO_2$ in the expired gases were made by the methods described by Wright *et al.* (1965).

Enzymic hydrolysis of the glucuronides of 2,6-dichloro-3- and -4-hydroxybenzonitrile. Solvent extracts of the urine of treated animals contained the glucuronides (R_F values on paper 0·15 and 0·45 in butan-1-ol–2 N-NH_3) of 2,6-dichloro-3- and -4-hydroxybenzonitrile (see the Methods and Results sections). Separate elution of individual zones from suitable chromatograms provided material for enzymic hydrolysis. After evaporation of the solvent 5 mg. of the residue was dissolved in 0·15 M-sodium acetate buffer, pH 5·0 (10 ml.), and incubated with β-glucuronidase (500 units) at 37° for 3 hr., and the mixture extracted with ether. 2,6-Dichloro-3- or -4-hydroxybenzonitrile was detected on thin-layer plates of the ether extract run in acetone–*n*-hexane (1:1, v/v) with phenoxyethanol–$AgNO_3$ reagent (Mitchell, 1958). Free glucuronic acid was detected with naphtharesorcinol (Tollens, 1908, 1910), and it was identified on paper chromatograms of the concentrated aqueous phase, run in butan-1-ol–acetic acid–water (4:1:1, by vol.), with naphtharesorcinol (Elliott, Parke & Williams, 1959) and *p*-anisidine hydrochloride (Hough, Jones & Wadman, 1950).

RESULTS

Excretion and retention of radioactivity in rats dosed respectively with [^{14}C]Prefix and 2,6-dichlorobenzo[^{14}C]nitrile. After oral treatment of six adult male and six adult female rats with either [^{14}C]Prefix or 2,6-dichlorobenzo[^{14}C]nitrile, the major eliminative route was via the kidneys and the remaining radioactivity was almost entirely eliminated by the faecal route. With the two ^{14}C-labelled herbicides, some ^{14}C was present in the gut of some of the animals at the end of 96 hr., especially when the animals were constipated on the third and fourth days (Tables 1 and 2). A single dose of either [^{14}C]Prefix or 2,6-dichlorobenzo[^{14}C]nitrile is almost entirely eliminated from rats in 4 days: 84·8–100·5% of ^{14}C from [^{14}C]Prefix and 85·8–97·2% of ^{14}C from 2,6-dichlorobenzo[^{14}C]nitrile are excreted, these values including the contents of the gut at 96 hr. Rats do not show a sex difference in the pattern of elimination of the respective metabolic products of either Prefix or 2,6-dichlorobenzonitrile.

Very small amounts of ^{14}C were found in the carcass and viscera remaining after removal of the gastrointestinal tract from animals dosed with [^{14}C]Prefix or 2,6-dichlorobenzo[^{14}C]nitrile. The values for ^{14}C in the carcass of animals dosed with [^{14}C]Prefix take into consideration the skin and hair (Table 1), whereas the skin plus hair of animals dosed with 2,6-dichlorobenzo[^{14}C]nitrile was assayed separately for ^{14}C.

Excretion of ^{14}C in the urines of rats dosed with either [^{14}C]Prefix or 2,6-dichlorobenzo[^{14}C]nitrile was very rapid, and the similar rates of elimination for the respective metabolic products of Prefix and 2,6-dichlorobenzonitrile in rat urine do not preclude the possibility that 2,6-dichlorobenzonitrile might be an intermediate in the metabolism of Prefix (Tables 3 and 4). Radioactivity was slowly

Table 1. *Excretion and retention of radioactivity in rats after oral administration of* [^{14}C]*Prefix*

Male and female rats (150–200 g. body wt.) were treated by stomach tube with 1·8 mg. of [^{14}C]Prefix (3·2 μc/mg.) in 2 ml. of aq. 5% ethanol.

		Recovery of ^{14}C during 4-day collection (% of dose)					
Rat no.	Sex	Faeces	Urine	Expired gases	Gut and contents	Carcass plus remaining viscera	Total
1	♂	20·4	68·0		0	0·4	88·8
2	♂	18·3	79·7		2·5*	0·5	101·0
3	♂	16·0	74·5	< 0·05	0	0·5	91·0
4	♂	17·1	74·8		0·5	0·5	92·9
5	♂	15·6	79·7		0·1	0·3	95·7
6	♂	17·1	67·3		0·4	0·4	85·2
7	♀	16·1	71·8		0	0·3	88·2
8	♀	18·0	71·4		0·4	0·2	90·0
9	♀	18·1	71·8	< 0·05	1·6	0·4	91·9
10	♀	20·9	74·7		0·7	0·3	96·6
11	♀	17·9	72·0		0·1	0·4	90·4
12	♀	9·2	76·0		8·6*	0·3	94·1

* Constipated animals (see Table 3).

Table 2. *Excretion and retention of radioactivity in rats after oral administration of 2,6-dichlorobenzo[^{14}C]nitrile*

Rats (150–200g. body wt.) were treated by stomach tube with 0·80mg. (males) or 1·3mg. (females) of 2,6-dichlorobenzo[^{14}C]nitrile (9·1μc/mg.) in 2ml. of arachis oil.

		Recovery of ^{14}C during 4-day collection (% of dose)						
Rat no.	Sex	Faeces	Urine	Expired gases	Gut and contents	Skin and hair	Carcass plus remaining viscera	Total
1	♂	19·0	74·3		0·1	0·1	0·1	93·6
2	♂	19·2	73·2		0·9	0·3	0·3	93·9
3	♂	18·5	72·7		0·1	0·1	0·1	91·5
4	♂	13·4	72·3	<0·02	0·1	0·2	0·2	86·2
5	♂	11·6	76·0		4·6	0·2	0·5	92·9
6	♂	19·1	74·6		1·2	0·2	0·2	95·3
7	♀	13·6	76·9		4·0	0·8	0·3	95·6
8	♀	15·1	80·7		0·6	0·6	0·2	97·2
9	♀	17·7	74·8		0·2	0·7	0·3	93·7
10	♀	16·3	78·6	<0·03	0·1	0·5	0·3	95·8
11	♀	19·7	77·3		0·2	0·5	0·2	97·9
12	♀	11·5	77·1		6·1*	0·5	0·3	95·5

* Constipated animal (see Table 4).

Table 3. *Rates of faecal and urinary elimination of radioactivity in rats after oral administration of [^{14}C]Prefix*

Male and female rats were treated with [^{14}C]Prefix as described in Table 1.

		Elimination of ^{14}C (% of dose)							
		0–24hr.		24–48hr.		48–72hr.		72–96hr.	
Rat no.	Sex	Faeces	Urine	Faeces	Urine	Faeces	Urine	Faeces	Urine
1	♂	15·3	66·1	4·7	1·3	0·3	0·4	0·1	0·3
2	♂	11·8	76·3	5·3	2·3	1·2	0·6	No faeces	0·5
3	♂	9·5	72·4	4·3	1·5	2·0	0·4	0·3	0·3
4	♂	13·8	72·1	2·6	1·8	0·3	0·6	0·4	0·4
5	♂	4·2	75·1	5·9	3·2	4·94	0·8	0·51	0·6
6	♂	10·4	65·2	1·5	0·5	3·8	1·1	1·3	0·5
7	♀	No faeces	70·0	15·4	0·9	0·5	0·6	0·1	0·4
8	♀	0·01	67·2	12·3	2·7	5·1	0·6	0·6	0·8
9	♀	9·6	67·5	3·6	2·5	1·7	0·8	3·2	1·0
10	♀	9·2	68·6	4·5	4·6	2·4	1·1	4·7	0·5
11	♀	No faeces	67·3	9·8	3·3	7·7	0·9	0·4	0·4
12	♀	6·0	71·2	3·2	3·6	No faeces	0·7	No faeces	0·5

eliminated in the faeces, and some irregularity in faecal elimination may be due to coprophagy. Alimentary absorption is virtually complete and the two herbicides are rapidly metabolized. A big proportion of the products of metabolism of each herbicide enters the circulating blood and is rapidly cleared in the kidneys; the low rate of elimination of metabolites in the faeces is probably due to enterohepatic circulation.

Elimination of radioactivity in dogs dosed with either [^{14}C]Prefix or 2,6-dichlorobenzo[^{14}C]nitrile. A single oral dose of either [^{14}C]Prefix or 2,6-dichlorobenzo[^{14}C]nitrile in dogs is almost entirely eliminated in 4 days: 85·9–106·1% of ^{14}C from [^{14}C]Prefix and 86·8–92·5% of ^{14}C from 2,6-dichlorobenzo[^{14}C]nitrile are excreted. The major eliminative route was via the kidneys and the remainder of the original label was almost entirely

Table 4. *Rates of faecal and urinary elimination of radioactivity in rats after oral administration of 2,6-dichlorobenzo[^{14}C]nitrile*

Male and female rats were treated with 2,6-dichlorobenzo[^{14}C]nitrile as described in Table 3.

		Elimination of ^{14}C (% of dose)							
		0–24 hr.		24–48 hr.		48–72 hr.		72–96 hr.	
Rat no.	Sex	Faeces	Urine	Faeces	Urine	Faeces	Urine	Faeces	Urine
1	♂	10·8	69·1	3·7	3·6	2·8	1·2	1·7	0·3
2	♂	1·5	63·6	14·1	7·7	2·4	1·3	1·3	0·6
3	♂	1·6	63·9	14·9	7·3	1·6	1·1	0·4	0·4
4	♂	9·5	66·7	3·3	2·6	0·4	1·2	0·2	1·8
5	♂	2·9	64·9	5·0	7·8	3·1	1·7	0·6	1·6
6	♂	5·9	67·6	12·4	3·8	0·4	1·0	0·4	2·2
7	♀	2·0	65·7	1·5	8·6	2·8	1·7	7·3	0·9
8	♀	No faeces	71·0	7·1	6·7	3·9	1·6	4·1	1·4
9	♀	4·0	68·5	5·7	4·1	5·2	1·5	2·8	0·7
10	♀	2·3	71·5	3·7	5·6	9·8	1·0	0·5	0·5
11	♀	4·4	70·0	6·4	6·0	6·3	0·9	2·6	0·4
12	♀	11·4	70·1	0·1	5·2	No faeces	1·1	No faeces	0·7

Table 5. *Rates of faecal and urinary elimination of radioactivity in male and female dogs after oral administration of either [^{14}C]Prefix or 2,6-dichlorobenzo[^{14}C]nitrile*

Each dog swallowed a gelatin capsule containing a solution of either 7·90 mg. of [^{14}C]Prefix (2·8 μc/mg.) in ethanol (1 ml.) or 0·92 mg. of 2,6-dichlorobenzo[^{14}C]nitrile (8·52 μc/mg.) in arachis oil (1 ml.).

			Elimination of ^{14}C (% of dose)							
			0–24 hr.		24–48 hr.		48–72 hr.		72–96 hr.	
Dog no.	Body wt. (kg.)	Sex	Faeces	Urine	Faeces	Urine	Faeces	Urine	Faeces	Urine
					[^{14}C]Prefix					
1	14·8	♂	No faeces	33·5	No faeces	18·5	24·4	11·9	1·6	2·7
2	14·5	♂	6·6	33·0	No faeces	16·2	9·2	14·6	2·2	4·1
3	10·4	♀	No faeces	55·1	No faeces	20·8	24·5	4·1	0·7	0·9
4	10·2	♀	0·6	51·3	3·8	18·2	No faeces	6·4	11·6	1·6
					2,6-Dichlorobenzo[^{14}C]nitrile					
5	11·5	♂	16·8	50·7	4·3	10·7	1·5	2·3	0·4	0·8
6	12·3	♂	15·1	44·8	10·2	11·2	1·5	2·9	0·4	1·1
7	12·7	♀	16·6	59·7	1·8	6·6	3·8	3·2	0·2	0·6
8	7·2	♀	11·2	44·1	5·3	13·7	2·6	6·2	1·4	2·3

eliminated by the faecal route (Table 5). Dogs do not show a sex difference in this pattern of elimination of the metabolites of either Prefix or 2,6-dichlorobenzonitrile. Dogs and rats do not show species differences in the patterns of elimination of the metabolic products of either Prefix or 2,6-dichlorobenzonitrile. Excretion of ^{14}C in the urines of treated dogs was slower than in rats. The elimination of ^{14}C in the urines of dogs treated with [^{14}C]Prefix was irregular and the rate of elimination of ^{14}C was different for the two sexes. This experiment was complicated by irregular defaecation of the animals, which were ill-adjusted to the diet, and the number of observations on the output of ^{14}C in the urine was too small for a conclusion on the difference in rate to be made.

Separation of fractions of chemically similar substances from the hydrolysed urines of rats dosed respectively with [^{14}C]Prefix and 2,6-dichlorobenzo-[^{14}C]nitrile. Rats treated with Prefix or 2,6-dichlorobenzonitrile do not eliminate either herbicide unchanged. 2,6-Dichlorobenzonitrile may be a metabilite of Prefix with a high turnover, since 2,6-dichlorobenzonitrile is itself completely metabolized in rats, and to test this supposition the metabolic products of the two herbicides were investigated.

By successive extraction at pH 1·5–2·0 with ether

and ethyl acetate, 80% of the radioactivity was recovered from the hydrolysed urines of rats dosed respectively with [^{14}C]Prefix and 2,6-dichlorobenzo[^{14}C]nitrile. In both cases the ethyl acetate extract contained one-half of the amount of ^{14}C found in the ether extract. Scanning revealed six radioactive zones on paper chromatograms of the ether-soluble material that had been run in butan-1-ol saturated with 2N-ammonia, and the fact that they reacted with phenoxyethanol–silver nitrate (Mitchell, 1958) was regarded as evidence that the main ^{14}C-labelled metabolite in each of the zones contained chlorine. These ether-soluble ^{14}C-labelled constituents were fractionated by column chromatography into aromatic acids, neutral constituents and phenols (Table 6). The ethyl acetate extracts of hydrolysed urines contained the more polar ^{14}C-labelled constituents, which also reacted with the phenoxyethanol–silver nitrate reagent.

A solution of each phenolic fraction in sodium hydroxide was purified by solvent extraction. Electrophoresis in veronal–hydrochloric acid buffer, pH 9·0, caused phenolic ^{14}C-labelled constituents to migrate towards the anode, whereas in acetate buffer, pH 5·2, they did not move. Major ^{14}C-labelled constituents that were separated by thin-layer chromatography were identified as 2,6-dichloro-3- and -4-hydroxybenzonitrile by their fluorescences in the ultraviolet and chromatographic properties (Table 7).

A solution of each neutral fraction was purified by extraction with 2N-sodium hydroxide, and electrophoresis of these fractions in buffers, pH 2·0–9·0, did not cause movement. A single ^{14}C-labelled constituent was identified as 2,6-dichlorobenzamide by its chromatographic properties (Table 7).

The acidic fractions were purified either by solvent extraction of their solution in aqueous sodium hydrogen carbonate or by thin-layer chromatography (Table 7). After electrophoresis in acetate

Table 6. *Separation of ether-soluble ^{14}C-labelled constituents of the hydrolysed urines into fractions of chemically similar substances*

Proportions of the ether-soluble material from the hydrolysed urines of rats (150–200g. body wt.) that had each been dosed with 1·8mg. of [^{14}C]Prefix (3·2μc/mg.) and 0·8mg. of 2,6-dichlorobenzo[^{14}C]nitrile (9·1μc/mg.) were chromatographed on a silicic acid column (10mm. diam. × 10cm. long). Recovery was quantitative.

Fraction no. (10 ml.)	Eluent	Recovery of radioactive constituents
1–3	20% Acetone in *n*-hexane	Phenolic fraction (80% of ^{14}C)
4–6	50% Acetone in *n*-hexane	Neutral fraction (1% of ^{14}C)
7–9	Acetone	Aromatic acids (19% of ^{14}C)

Table 7. *Fluorescences, R_F values on paper and thin plates, and retention times on gas–liquid chromatographs for the ether-soluble constituents in hydrolysed urines of rats dosed with either Prefix or 2,6-dichlorobenzonitrile*

The labelled constituents were identified by comparing their chromatographic and fluorescence properties with those of the authentic unlabelled compounds.

Constituents in hydrolysed urines	R_F: Paper in butan-1-ol satd. with 2N-NH_3	R_F: Thin-layer plates in acetone–*n*-hexane (1:1, v/v)	Fluorescence in u.v.	Retention times for the methylated derivatives* (min.)	
2,6-Dichlorobenzamide	0·92	0·7	Dull violet	15·0	(*a*)
2,6-Dichloro-3-hydroxybenzonitrile	0·66	0·9	Bright violet	4·0	(*a*)
2,6-Dichloro-4-hydroxybenzonitrile	0·72	0·7	Dull violet	3·5	(*a*)
2,6-Dichlorobenzoic acid	0·45	0·02	Dull violet	4·0	(*b*)
2,6-Dichloro-3-hydroxybenzoic acid	0·06	0·02	Dull violet	17·6	(*b*)
Unknown aromatic acid (2,6-Dichloro-4-hydroxybenzoic acid)	0·16	0·02	—		

* After their separation, the acid, the two phenols and the phenolic acid respectively were exhaustively methylated with excess of diazomethane in ether solution, and *n*-hexane solutions of the methylated derivatives were used for gas–liquid chromatography: (*a*) Stationary phase: 5% (w/w) phenyl diethanolamine succinate on Celite; flow rate, 50ml./min.; temperature, 188°. (*b*) Stationary phase: 3·8 (w/w) SE30 silicone oil on Diatopore S; chromatograph not equipped with flow-meter; temperature, 120°.

buffer, pH 5·2, acidic ^{14}C-labelled constituents migrated towards the anode. Major ^{14}C-labelled components in the acidic fractions that were separated by paper chromatography were identified as 2,6-dichlorobenzoic acid and 2,6-dichloro-3-hydroxybenzoic acid by their chromatographic properties (Table 7). An unidentified ^{14}C-labelled component was possibly 2,6-dichloro-4-hydroxybenzoic acid. After electrophoresis in veronal–hydrochloric acid buffer, pH 9·0, 2,6-dichloro-3-hydroxybenz[^{14}C]oic acid migrated twice as far as 2,6-dichlorobenz[^{14}C]oic acid.

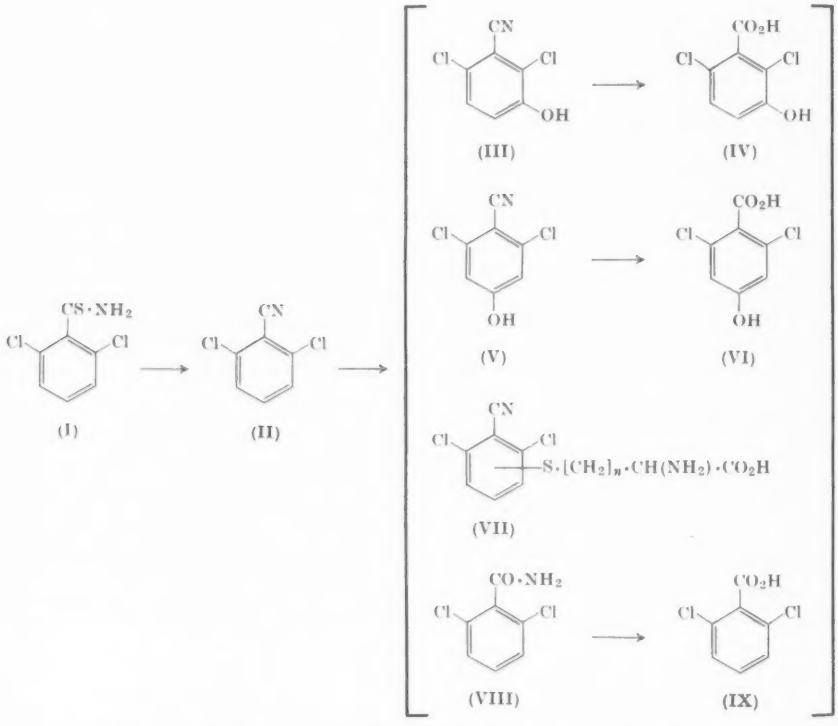
Polar ^{14}C-labelled constituents, which account for the radioactivity in the residual aqueous phase after ether extraction, could be quantitatively extracted with butan-1-ol, but the resulting solution was contaminated with large amounts of the components of normal urine, including electrolytes and water. A solution, containing major proportions of these ^{14}C-labelled constituents and smaller amounts of the contaminating substances, especially electrolytes and water, was prepared by exhaustive extraction with ethyl acetate; four main radioactive zones and two minor ones were present on paper chromatograms. At least one ^{14}C-labelled component was adsorbed on to a column of cation-exchange resin in the H^+ form and eluted with basic buffers. Electrophoresis (120 v/cm.) in acetic acid–formic acid buffer, pH 2·0, caused a single radioactive peak to migrate towards the cathode with one-half of the speed of glycine. In acetate buffer, pH 5·2, there was no movement in an electric field, and electrophoresis in veronal–hydrochloric acid buffer, pH 9·0, caused a single radioactive peak to move towards the anode. The presence of at least one deacetylated [^{14}C]mercapturic acid in the hydrolysed urines would account for these observations, and this supposition was tested by labelling the cysteine–cystine pools of rats with ^{35}S and treating these animals with the unlabelled herbicides (see the Methods section; Gutmann & Wood, 1950; Marsden & Young, 1958). This experiment provides clear evidence for the presence in the hydrolysed urines of two ^{35}S-labelled constituents, R_F 0·37 and 0·47 on paper. These ^{35}S-labelled constituents were absent from paper chromatograms of the hydrolysed urine of the control animals that had been given [^{35}S]cystine. Neither of these ^{35}S-labelled constituents could have been confused with $^{35}SO_4^{2-}$, which ran entirely differently in the butan-1-ol–2N-ammonia solvent system employed, and neither of them was due to organic [^{35}S]sulphate esters which would have been hydrolysed by the acid hydrolysis. These ^{35}S-labelled hydrolysis products were ninhydrin-positive, and they migrated in an electric field (120 v/cm.) in acetic acid–formic acid buffer, pH 2·0, towards the cathode and in veronal–hydrochloric acid buffer, pH 9·0, towards the anode. The foregoing chromatographic and electrophoretic properties of the ^{35}S-labelled constituents corresponded to those of two ^{14}C-labelled constituents present in the hydrolysed urines of animals dosed with the ^{14}C-labelled herbicides. The conclusion is drawn that the hydrolysed urines of animals dosed with either [^{14}C]Prefix or 2,6-dichlorobenzo[^{14}C]-nitrile and the hydrolysed urines of animals treated with [^{35}S]cystine and the unlabelled herbicides contained two sulphur-containing constituents derived from metabolite(s) of the two herbicides, and that the physical and chemical properties of these constituents are consistent with the possession of an associated amino acid residue. From the conspectus of knowledge on the mammalian metabolism of foreign organic compounds, it is known that there are two common types of sulphur-containing metabolites, mercapturic acids and sulphate esters (see Young & Maw, 1958). For the hydrolytic products, we are not concerned with sulphate esters and the sulphur-containing compounds in the hydrolysed urines may have been produced from mercapturic acid(s) or premercapturic acid(s) by hydrolysis. The nature of the amino acid residue in each sulphur-containing constituent has not been investigated, and the structures of the two sulphur-containing constituents have not been elucidated; this would require the isolation of pure compounds followed by careful degradative studies. However, our knowledge of the chemistry of the two herbicides indicates that the chloro substituents would not be expected to undergo nucleophilic replacement, and that the reactivities of the cyano group of 2,6-dichlorobenzonitrile and of the thioamido group of Prefix are very hindered indeed. Thus the addition of hydrogen sulphide to 2,6-dichlorobenzonitrile takes place under forcing conditions, whereas the addition of mercaptans does not take place under any conditions (Dr J. Yates, personal communication). The two sulphur-containing compounds in hydrolysed urines of treated animals may therefore be substituted 2,6-dichlorobenzonitriles, and this was tested by dethiolation with Raney nickel catalyst, when each of the sulphur-containing constituents generated a compound with the chromatographic properties of 2,6-dichlorobenzonitrile. The data seem to be consistent with the partial-type formula (VII) (Scheme 1) suggested for these compounds.

In the work-up of the hydrolysed urine from animals dosed with the ^{14}C-labelled herbicides, the previously mentioned ^{14}C that remained in the aqueous phase after exhaustive extraction with ether and ethyl acetate, and that could be completely extracted with butan-1-ol, was shown to be almost entirely due to further amounts of the two sulphur-containing constituents, R_F 0·37 and 0·47

Table 8. *Proportions of* ^{14}C*-labelled constituents in the hydrolysed urines of rats dosed respectively with* [^{14}C]*Prefix and 2,6-dichlorobenzo*[^{14}C]*nitrile*

The labelled constituents were identified by comparing their chromatographic and fluorescence properties with those of the authentic unlabelled compounds.

Constituents in hydrolysed urine	Rats dosed with ...	Proportions of ^{14}C-labelled metabolites (% of total ^{14}C in the urines) [^{14}C]Prefix	2,6–Dichlorobenzo-[^{14}C]nitrile
2,6-Dichlorobenzamide		<1·0	<1·0
2,6-Dichlorobenzoic acid		2·3	<1·0
2,6-Dichloro-3-hydroxybenzoic acid		2·8	5·0
2,6-Dichloro-3-hydroxybenzonitrile		42·0	41·7
2,6-Dichloro-4-hydroxybenzonitrile		2·8	4·0
Unknown aromatic acid (R_F 0·16)		2·1	1·3
Ethyl acetate-soluble metabolites: (I)		3·0	3·0
(II)		4·9	6·8
(III)		8·1	9·0
(IV)		9·4	6·5
(V)		<1·0	2·2
(VI)		<1·0	1·5
Recovery ...	...	>77·4	>81·0



Scheme 1. Transformations of Prefix and 2,6-dichlorobenzonitrile in the rat.

on paper. Greater proportions of these compounds than those shown in Table 8 were therefore present in the hydrolysed urines, from which they were incompletely extracted with ethyl acetate. The two sulphur-containing constituents are accordingly important to the transformations of Prefix and 2,6-dichlorobenzonitrile in the rat.

The structures of the ^{14}C-labelled constituents that have been investigated in the hydrolysed urine are related to those of the two herbicides in such a way that they may have been formed direct from the ^{14}C-labelled metabolites by hydrolysis.

Relative proportions of the various constituents in the hydrolysed urine of rats dosed with Prefix and 2,6-dichlorobenzonitrile. Quantitative aspects of the comparative metabolism of Prefix and 2,6-dichlorobenzonitrile in rats are summarized in Table 8. Pooled urines of 12 rats dosed with [^{14}C]Prefix and pooled urines of 12 rats dosed with 2,6-dichlorobenzo[^{14}C]nitrile were separately hydrolysed in the way that has been described, and radioactivity corresponding to the different ^{14}C-labelled constituents present in the various fractions was measured. These results were reproducible, the experiment being repeated with two further sets of 24 rats with identical results. Metabolism of the two herbicides yields the same constituents in the hydrolysed urine in strikingly similar proportions. For Prefix (I) (Scheme 1), *m*-hydroxylation accounts for 44·8% of the constituents (III and IV), and, on the assumption that the unknown aromatic acid is 2,6-dichloro-4-hydroxybenzoic acid (VI), *p*-hydroxylation accounts for 4·9% of the constituents (V and VI). For 2,6-dichlorobenzonitrile (II), the corresponding values are 46·7 and 5·3%. Metabolism of the two herbicides also yields approximately similar proportions of polar constituents, of which two of the ethyl acetate-soluble constituents are sulphur-containing compounds of partial-type formula (VII).

Since Prefix and 2,6-dichlorobenzonitrile have essentially similar biological properties and herbicidal activities (Barnsley, 1960), and since 2,6-dichlorobenzonitrile is the main metabolite of Prefix in plants and unsterilized soil (Barnsley, 1960; Milborrow, 1965), the possibility arises that Prefix might be transformed into 2,6-dichlorobenzonitrile in animals, and the fact that the metabolism of the two herbicides has given the same constituents in the hydrolysed urine in strikingly similar proportions strongly suggests an interrelationship of the metabolic pathways of the two herbicides. Prefix therefore appears to be extensively transformed into 2,6-dichlorobenzonitrile:

$$R\cdot CS\cdot NH_2 \rightarrow R\cdot CN + H_2S$$
where R = $Cl_2C_6H_3$

There is slight evidence that the competitive reactions:

$$R\cdot CS\cdot NH_2 + H_2O \rightarrow R\cdot CO\cdot NH_2 + H_2S$$
$$R\cdot CO\cdot NH_2 + H_2O \rightarrow R\cdot CO_2H + NH_3$$

take place to a very limited extent, since the hydrolytic product, 2,6-dichlorobenzoic acid (IX) (Scheme 1), is formed *in vivo* in greater quantity from Prefix than from 2,6-dichlorobenzonitrile; but the same quantity of 2,6-dichlorobenzamide was measured in parallel experiments with the two herbicides.

The results are not consistent with direct hydroxylation of Prefix and subsequent hydrolysis of thioamido products. The stability of 2,6-dichloro-3-hydroxythiobenzamide to boiling 6N-hydrochloric acid, and its absence from the hydrolysed urines of animals dosed with Prefix, is strong evidence that hydroxylated thiobenzamides were not formed *in vivo*.

Scheme 1 shows the transformations of Prefix and 2,6-dichlorobenzonitrile in rats.

^{14}C-labelled metabolites in the unhydrolysed urines of rats dosed respectively with [^{14}C]*Prefix and* 2,6-*dichlorobenzo*[^{14}C]*nitrile.* Free 2,6-dichloro-3- and -4-hydroxybenzonitrile were extracted in small quantity from the urine of rats dosed with either Prefix or 2,6-dichlorobenzonitrile. These phenols may have been excreted as their sulphate esters, which would probably have been hydrolysed under the prevailing acidic conditions of our ether extraction (see Young & Maw, 1958). In other experiments, urine at pH 6·0 was extracted with ether, and the material in the residual aqueous phase was submitted to paper electrophoresis (13 v/cm.) in 0·025M-phthalate buffer, pH 5·9, for various times. The rapidly migrating radioactive area from +14 to +20 cm. (Fig. 1) of strongly anionic substances was tentatively ascribed to strongly acidic sulphate esters; in another context similar assignments have also been made (Williams, Meikle & Redemann, 1964). The relative proportion of strongly anionic substances, when urines were extracted at pH 6·0, was almost the same as that of the free phenols, when the urines were extracted under more acid conditions.

Glucuronides of 2,6-dichloro-3- and -4-hydroxybenzonitrile had R_F 0·15 and 0·45 on paper in the butan-1-ol–2N-ammonia solvent system. Such compounds were also present in the urine of treated animals, and separation by paper chromatography followed by hydrolysis of individual components with β-glucuronidase generated 2,6-dichloro-3- and -4-hydroxybenzonitrile and free glucuronic acid (for details see the Methods section).

The aromatic acids that had previously been found in the hydrolysed urines of rats dosed with the two herbicides appear to be present as ester

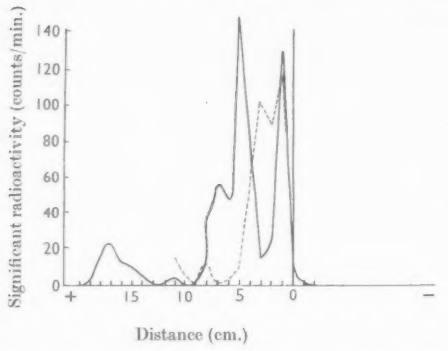


Fig. 1. Electrophoretic separation of ^{14}C-labelled metabolites in the residual aqueous phase after ether extraction of urine at pH 6·0. Metabolites were separated from the residual aqueous phase by electrophoresis on Whatman no. 1 filter papers in 0·025 M-phthalate buffer, pH 5·9, at 13 v/cm. for 2·5 hr. (——) or 1·0 hr. (– – – –).

glucuronides in the original urines. Hydrolysis with β-glucuronidase caused the disappearance of radioactive spots from the paper chromatograms of ether-extracted urines and the simultaneous generation of other radioactive spots due to the ^{14}C-labelled aromatic acids that had been found in the hydrolysed urines.

The urine of rats in which the cysteine–cystine pools had been labelled with ^{35}S and where the animals were dosed with unlabelled herbicides contained two ^{35}S-labelled metabolites that were absent from the urine of control rats that had been given [^{35}S]cystine. One of these substances could be extracted by ether (R_F 0·20 on paper in butan-1-ol–2 N-ammonia) and the other by ethyl acetate (R_F 0·27 on paper in butan-1-ol–2 N-ammonia). These metabolites did not migrate in an electric field (120 v/cm.) towards the cathode in acetic acid–formic acid buffer, pH 2·0, and they did not give a colour reaction with ninhydrin. The chromatographic and electrophoretic properties of the foregoing ^{35}S-labelled metabolites corresponded to those of two ^{14}C-labelled metabolites present in the urines of rats dosed with the ^{14}C-labelled herbicides. The conclusion is drawn that the urines of rats dosed with [^{14}C]Prefix or 2,6-dichlorobenzo[^{14}C]nitrile contained two sulphur-containing metabolites. Hydrolysis of these metabolites with 6 N-hydrochloric acid gave the two ^{14}C-labelled sulphur-containing amino acid constituents, which migrated in an electric field towards the cathode in buffers of pH 2·0 and towards the anode in buffers of pH 9·0. The generation of a single sulphur-containing amino acid from each of the sulphur-containing metabolites, in the way that has been described, may have been brought about by elimination of an acyl group from the amino nitrogen atom of each sulphur-containing acylamino acid metabolite. The conditions selected for hydrolysis of the urine were those that would have been sufficiently drastic to effect *N*-deacetylation of *N*-acetylcysteyl-containing metabolites (Thomson, Barnsley & Young, 1963). The structures of the two sulphur-containing metabolites have not been elucidated; at first sight, they may be analogous to 2,6-dichlorobenzonitrile mercapturic acids or premercapturic acids.

Quantitative assessments were made on the unhydrolysed urines of rats dosed with [^{14}C]Prefix and 2,6-dichlorobenzo[^{14}C]nitrile to give information for whole urines of the sort reported in Table 8 for hydrolysed urines. Not much would be gained by the inclusion of detailed information, since reference would have to be made to unknown metabolites and partially elucidated metabolites. Valuable confirmation was obtained of the amounts of ^{14}C-labelled constituents present in the various solvent extracts of hydrolysed urines by the preparation of solvent extracts of hydrolysates of the corresponding solvent extracts of whole urine.

DISCUSSION

Treatment of animals with Prefix or 2,6-dichlorobenzonitrile has given rise to the expected metabolic products.

3- and 4-Hydroxylation of 2,6-dichlorobenzonitrile has now been demonstrated *in vivo*; hydroxylation is predominantly *meta*, owing to the inductive effect of the 2,6-dichloro atoms. The 2,6-dichloro substituents contribute to the rapid rate of hydroxylation of 2,6-dichlorobenzonitrile *in vivo*, since the slowness with which the metabolic products of benzonitrile and nitrobenzene are eliminated from the body has been attributed to difficulty associated with the hydroxylation of aromatic compounds that have *meta*-directing groups (Bray, Hybs & Thorpe, 1951; Smith, 1950; Smith & Williams, 1950). By analogy with chlorobenzene (Smith, Spencer & Williams, 1950) and naphthalene (Corner & Young, 1955), the possibility that hydroxylation of 2,6-dichlorobenzonitrile *in vivo* may involve intermediate formation of 2,6-dichloro-3,4-dihydro-3,4-dihydroxybenzonitrile is attractive, since this diol might itself be formed from an epoxide precursor, which would also be expected to react with the thiol group of cysteine, glutathione or tissue proteins (Parke & Williams, 1958) to yield the two sulphur-containing acylamino acid metabolites that have been discussed.

In general, arylnitriles are metabolized to only a

small extent by reactions involving the cyano group:

$$Ar{\cdot}CN \rightarrow Ar{\cdot}CO{\cdot}NH_2 \rightarrow Ar{\cdot}CO_2H$$

The reaction is a minor one, and its extent depends on the nature of the substituents in the benzene nucleus. With benzonitrile and *o*-tolunitrile, oxidation of the aromatic nucleus is the major pathway of metabolism, but at least 10% of benzonitrile and 25% of *o*-tolunitrile are metabolized by hydrolysis of the cyano group to carboxyl (Bray *et al.* 1951). Less than 2% of the sterically hindered 2,6-dichlorobenzonitrile is metabolized *in vivo* to 2,6-dichlorobenzamide plus 2,6-dichlorobenzoic acid. These compounds cannot be produced *in vitro* by drastic acid hydrolysis, and it is noteworthy that the amide is stable to prolonged refluxing with 6N-hydrochloric acid. The hydroxylation products, 2,6-dichloro-3- and -4-hydroxybenzonitrile, are also partially hydrolysed *in vivo* to 2,6-dichloro-3- and -4-hydroxybenzoic acid.

In agreement with the known behaviour of other arylnitriles, no evidence was found for the formation of hydrogen cyanide from 2,6-dichlorobenzonitrile *in vivo*. Thus when 2,6-dichlorobenzonitrile was administered orally, LD_{50} values (expressed as mg./kg.) were more than 3000 (rats), 866 (mice) and more than 1250 (chickens). The toxicological properties and symptomatology of acute poisoning are incompatible with release of hydrogen cyanide.

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The Isolation and Partial Characterization of Glycolipids of Normal Human Leucocytes

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1. The lipids of purified human leucocytes were extracted with chloroform–methanol and the extract was washed with water. Glycolipids, isolated by Florisil chromatography, were subjected to mild alkaline hydrolysis and the alkali-resistant fraction was fractionated on a silicic acid column. 2. Three classes of glycolipid were separated. The less polar, containing 3·6% of the total glycolipid hexose as galactose, was tentatively identified as ceramide monohexoside. The major glycolipid fraction was characterized as ceramide dihexosides. The more polar glycolipids comprised 1·6% of the total glycolipid hexose as galactose and glucose (in the molar ratio 2:1) and were non-acidic. This class was separated as a mixture containing ninhydrin-positive glycolipids. 3. The ceramide dihexosides taken from two leucocyte preparations accounted for 15·2% and 16·4% by weight of the total lipids. 4. The carbohydrate moiety of the ceramide dihexosides contained galactose and glucose in the molar ratio 2:1. Partial acid hydrolysis and paper chromatography indicated that the hexoses are present as disaccharides, lactose being identified as one of them. 5. Palmitic acid ($C_{16:0}$) and nervonic acid ($C_{24:1}$) were the major fatty acids of this glycolipid. Hydroxy fatty acids were not detected.

Cerebroside and cerebroside sulphate, containing in their molecule 1 hexose moiety/amino base, are the most common water-insoluble glycolipids of the mammalian nervous system, from which they have mostly been isolated and characterized.

Similar substances, with two hexose moieties/molecule, occur in small quantities in various tissues (Klenk & Rennkamp, 1942; Rapport, Graf, Skipski & Alonzo, 1959; Yamakawa, Kiso, Handa, Makita & Yokoyama, 1962; Svennerholm & Svennerholm, 1963; Gray, 1965). They have all been identified as ceramide dihexosides containing both glucose and galactose, usually in equal proportions, and they occur together with other water-insoluble glycolipids as minor components of the total lipids of the tissue. When examined immunologically, they show a biological (haptenic) activity that has been correlated with their molecular structure (Rapport, 1960). Some of the ceramide dihexosides contained hydroxy fatty acids as well as the normal straight-chain fatty acids.

As a continuation of our work on the lipid metabolism of normal and leukaemic leucocytes (Malamos, Miras, Levis & Mantzos, 1962; Miras, Mantzos & Levis, 1965), we have studied the water-insoluble glycolipids of these cells.

EXPERIMENTAL

Isolation of leucocytes. Blood from healthy male blood donors, who had not eaten for 12hr., was used as a source of leucocytes. Blood (4 vol.) was aspirated into 1 vol. of anti-coagulant solution (0·48% citric acid hydrate–1·32% sodium citrate–1·47% dextrose).

Sedimentation of erythrocytes was carried out as described by Malamos *et al.* (1962), yielding leucocytes contaminated with fewer than 4 erythrocytes/leucocyte. To achieve purer preparations, the leucocytes were re-suspended in a small volume of plasma (free of platelets) and erythrocytes were added (taken from the first sediment). At this stage leucocytes were present in a concentration approx. 80 times that of the original blood, erythrocytes having half the normal concentration. Then polyvinyl-pyrrolidone solution was added to give a final concentration of 1·5% and the suspension was mixed gently. The leucocytes were then separated and washed twice with 10ml. of 0·85% NaCl solution. The purified preparation contained approx. 75% of the blood leucocytes contaminated with 2–5 erythrocytes and 10–20 platelets/100 leucocytes. The differential count was not altered significantly.

Extraction of lipid. The lipid was extracted by a slight modification of the method of Folch, Lees & Sloane-Stanley (1957). To 1 vol. of leucocyte suspension in water, 6·3 vol. of methanol was added, and the mixture was shaken vigorously for 20 min.; 12·6 vol. of chloroform was then added and the mixture shaken for 40 min. The mixture was filtered and the procedure of Folch *et al.* (1957) was applied

to the filtrate with only one wash with the theoretical upper phase.

Isolation of the glycolipid fractions. The total lipids (30–40 mg.) were dissolved in chloroform and placed on a Florisil (Floridin Co., Tallahasse, Fla., U.S.A.) column (5g.), the elution being carried out successively with chloroform, chloroform–methanol (19:1, v/v), chloroform–methanol (2:1, v/v) and chloroform–methanol (1:2, v/v). The anthrone test was performed on small samples of the eluted fractions to identify the glycolipids. Each of the two last fractions contained the glycolipids and was treated with a mixture containing equal volumes of chloroform and 0·6N-NaOH in methanol (Hübscher, Hawthorne & Kemp, 1960; as applied by Sweeley, 1963) for 1hr. at room temperature and was then acidified and extracted with chloroform. The chloroform phase was dried and placed on a silicic acid column (2g.) (special for lipid chromatography; Bio-Rad Laboratories, Richmond, Calif., U.S.A.). Methyl esters of fatty acids formed during the methanolysis were eluted from the column with chloroform. The elution was then carried out successively with the following mixtures of chloroform–methanol: (19:1, v/v), (18:2, v/v) and (16:4, v/v). Column chromatography on DEAE-cellulose was occasionally used according to the method of Rouser, Baumann, Kritchevsky, Heller & O'Brien (1961) for identification of acidic glycolipids.

Thin-layer and paper chromatography. Basic and neutral plates, prepared according to the method of Skipski, Peterson & Barclay (1962), were used for thin-layer chromatography, the first being developed with chloroform–methanol–acetic acid–water (70:12:3:2, by vol.) and the second with chloroform–methanol–aq. NH_3 (sp.gr. 0·88) (80:20:0·4, by vol.).

The spots were made visible by spraying with 50% (v/v) H_2SO_4 or with other reagents (Mangold, 1961). Thin-layer chromatography was also performed on plates with DEAE-cellulose (Mannex DEAE; Mann Research Laboratories Inc., New York, N.Y., U.S.A.) and developed with chloroform–methanol (2:1, v/v); spots were made visible by spraying with bromophenol blue. The lipid bases were chromatographed on thin-layer plates according to the method of Fujino & Zabin (1962). Fatty acid esters were chromatographed on neutral plates with light petroleum (b.p. 40–60°)–diethyl ether–acetic acid (40:10:1, by vol.).

Sugars were identified in the hydrolysate by descending paper chromatography on Whatman no. 3 paper in butan-1-ol–acetic acid–water (4:1:5, by vol.) and pyridine–butan-1-ol–water (1:2:1, by vol.). Spots were revealed with aniline hydrogen phthalate or $AgNO_3$ reagents.

Hexose determination. Hexose was determined with anthrone in two different non-hydrolysed samples, by the method of Radin, Lavin & Brown (1955), on 150 and 200 μg. samples; a sample of brain cerebroside, purified by chromatography on a Florisil column and checked for purity by thin-layer chromatography and infrared analysis, was used as a standard. Analysis of total nitrogen and total hexose as galactose of this standard showed a galactose/nitrogen molar ratio 1:0·95.

Hexose was determined after hydrolysis as follows: the glycolipid (approx. 2mg.) was hydrolysed with 4ml. of 3N-HCl at 100° for 3hr. After removal of the HCl under reduced pressure, the residue was taken up in water and several samples were used for the estimation of the total hexose and of the galactose/glucose ratio by the carbazole method, as described by Seibert & Atno (1946). Hexose determination with anthrone was performed on the hydrolysate by the method of Radin, Brown & Lavin (1956).

Glucose was determined enzymically with glucose oxidase (Huggett & Nixon, 1957).

Identification of fatty acids. The glycolipid was hydrolysed by refluxing for 6hr. with methanol–conc. HCl (5:1, v/v). The fatty acids were extracted with light petroleum (b.p. 40–60°), esterified and analysed by gas–liquid chromatography. A Pye Argon gas chromatograph fitted with a polyethylene glycol adipate (10%, by wt., on Celite) column was used and the peaks were identified by comparison with standard methyl esters prepared from brain cerebrosides (Mead & Levis, 1963).

Determination of long-chain base. After the extraction of the fatty acids from the hydrolysate, most of the methanol was removed in a stream of N_2, water was added and the solution was made alkaline with conc. KOH. The long-chain bases were extracted into ethyl acetate and washed with water, and sphingosine was determined quantitatively by the method of Lauter & Trams (1962). The lipid base fraction of a brain cerebroside sample, with known nitrogen content, was used as a standard.

Partial hydrolysis. The glycolipid was heated in a boiling-water bath with 0·1N-HCl for 1hr. with continuous stirring. The hydrolysate was extracted with chloroform and the extract was subjected twice more to the same procedure, and then twice to the same procedure with 0·2N-HCl. The unaffected lipids were chromatographed on a silicic acid column with chloroform and chloroform–methanol (19·5:0·5, v/v), (19:1, v/v) and (18:2, v/v) as eluting solvents. The water phases from the hydrolysis steps after removal of the HCl under reduced pressure were first analysed individually for total hexose and were then combined and used for glucose estimation and paper-chromatographic analysis of the carbohydrates.

Nitrogen and phosphorus determination. Nitrogen was determined by direct nesslerization of the digested lipid by the method of King, Haslewood & Delory (1937). Phosphorus was determined by the method of King (1932) with the volumes adjusted to measure 0·4 μg. of phosphorus.

Infrared analysis. Infrared analysis was performed on KBr pellets with a Beckam IR–5 spectrophotometer.

RESULTS

Two preparations, each extracted from the leucocytes of approx. 1·2l. of blood taken from three individuals, contained (I) 35mg. and (II) 28mg. of lipid.

The alkali-stable lipids, eluted from the Florisil column with chloroform–methanol (2:1, v/v), were chromatographed on a silicic acid column. A small glycolipid fraction was eluted with chloroform–methanol (19:1, v/v). The hexose content of the fraction, measured as galactose, accounted for 3·6% of the hexose of the total glycolipids. On thin-layer chromatography it showed the same mobility as brain ceramide galactoside; it was therefore tentatively identified as ceramide monohexoside. Further analysis of this fraction was not possible because of lack of material. Most of the glycolipid

was eluted as a single peak with chloroform–methanol (18:2, v/v). The yields were 5·3mg. and 4·6mg. of lipid from preparations I and II respectively.

The glycolipid was a white powder (phosphorus content less than 0·02%) that reacted strongly with anthrone reagent and did not show any reducing properties. On thin-layer chromatography it appeared as a double spot, negative to ninhydrin.

The glycolipid, when chromatographed on a DEAE-cellulose column, was eluted in the non-acidic fraction with chloroform–methanol (2:1, v/v). On thin-layer plates, coated with DEAE-cellulose, it moved with the solvent front, whereas a sample of brain cerebroside sulphate run simultaneously remained at the origin. The infrared spectrum showed features of the cerebroside molecule (Marinetti & Stotz, 1954), being almost identical with those presented for the blood serum ceramide dihexoside (Svennerholm & Svennerholm, 1963). The glycolipid did not contain any sulphate, as indicated by the absence of the characteristic bands for S–O and C–O–S at 1240 and 820cm.$^{-1}$ respectively (Lloyd & Dodgson, 1961). A weak band near 1720cm.$^{-1}$, possibly attributable to ester group, remained unchanged after mild alkaline hydrolysis and chromatography on silicic acid.

Paper chromatography of the carbohydrate fraction after complete acid hydrolysis revealed two spots with mobilities identical with those of glucose and galactose. After partial hydrolysis with 0·1N-hydrochloric acid two spots with the mobilities of galactose (main spot) and lactose were obtained; glucose could only be identified by the enzymic method and accounted for 3·4% of the total liberated hexose. After treatment with 0·2N-hydrochloric acid the chromatogram showed three spots with the mobilities of glucose, galactose and lactose, the third being faintly positive; glucose accounted for 24·5% of the total liberated hexose. The identity of the lactose spot was further established by two-dimensional paper chromatography. In this instance the hydrolysate was chromatographed after [1-^{14}C]lactose was added to it. Radioautographs taken after solvent development showed complete coincidence of the radioactive and colour spots of lactose. The lipid fraction was chromatographed on a silicic acid column after partial acid hydrolysis and, in addition to unchanged material, a new glycolipid fraction was eluted with chloroform–methanol (19:1, v/v). On thin-layer chromatography the new glycolipid fraction appeared as a double spot, with mobilities similar to those of ceramide monohexosides. These findings indicate the presence of both glucose and galactose bound in the form of dihexosides. The easier liberation of galactose compared with that of glucose indicates that the glucose-containing dihexoside is linked to the ceramide through glucose.

Table 1 shows the results of chemical analysis of the glycolipid. Determination of the fatty acid components was performed on different portions of one sample, derived by pooling together the remainders of each preparation after estimation of hexose and nitrogen.

The results of anthrone analysis on non-hydrolysed samples are expressed as the ratios of the extinctions/g.atom of nitrogen for the colours produced by the leucocyte glycolipid and the ceramide galactoside standard. The data of the first column of Table 1 could suggest that the leucocyte glycolipid contains more than 2 moles of galactose/g.atom of nitrogen, but since glucose with anthrone produce about 1·8 times more colour than galactose the presented ratios are close to those expected for a dihexoside containing galactose and glucose in the molar ratio 2:1. This finding correlates well with the glucose content of the glycolipid, which was determined in the hydrolysate by both the carbazole and glucose-oxidase methods.

Estimation of total hexose in the hydrolysates of the sample glycolipid and of the standard brain cerebroside by the anthrone method showed that the total hexose/nitrogen ratio of the hydrolysed

Table 1. *Chemical analysis of human leucocyte ceramide dihexoside*

The ratios of *E*(anthrone)/g.atom of nitrogen for leucocyte glycolipid to that for ceramide galactoside standard are averages of duplicate hexose determinations with anthrone on two different non-hydrolysed samples with known nitrogen content. Total hexose was determined by the carbazole method after hydrolysis; a mixture of galactose and glucose (molar ratio 2:1) was used as standard. Sphingosine was determined in a mixture of glycolipids of preparations I and II.

	Ratio of *E*(anthrone)/g.atom of N (leucocyte glycolipid/ceramide galactoside)	Total hexose (% dry wt.)	Glucose (% of total hexose)		Total N (% dry wt.)	Sphingosine/total N molar ratio
			Carbazole method	Enzymic method		
Preparation I	2·43	32·6	34	33	1·38	0·85
Preparation II	2·37	31·5	35·5	34·5	1·45	

sample was the same as that found in the non-hydrolysed one. This result indicates that both sample and standard were hydrolysed to the same extent. The actual recovery of total hexose, as judged from galactose estimation in triplicate hydrolysates of the standard, was $85 \pm 3\%$. When this recovery is taken into consideration, it is obvious that the data for total hexose presented in Table 1 are near to 38·8%, which is the value for hexose expected in a ceramide dihexoside containing fatty acids with an average mol.wt. of 320. The calculation of this value was based on the fatty acid composition derived from gas–liquid-chromatographic analysis.

The lipid base fraction has not been definitively identified, but it shows on thin-layer chromatography one major and one less intense ninhydrin-positive spot, with mobilities identical with those of a corresponding fraction from brain cerebrosides. The amount of sphingosine, estimated after hydrolysis, was less than 1 mole/g.atom of total nitrogen (Table 1). This may be due to partial removal of sphingosine into the fatty acid fraction.

Gas–liquid chromatography of the fatty acid methyl esters revealed the presence of only normal fatty acids (Table 2).

The leucocyte ceramide dihexoside appeared as a double spot on thin-layer chromatography. The presence of α-hydroxy fatty acids could probably explain a slower-running spot (Jatzkewitz, 1960), but as such fatty acids were not found in the hydrolysate of the glycolipids the two spots were separated by preparative thin-layer chromatography and analysed for glucose content, for fatty acid composition and for lipid base. The glucose content and the lipid base profiles were the same, but the fatty acid compositions of the two spots showed some differences (Table 2). The faster-running spot contained predominantly the longer-chain fatty acids, whereas palmitate was the predominant fatty acid in the slower spot. There was not a clear-cut distinction between the fatty acid compositions of the two spots, owing to a small overlap that occurred during the preparative thin-layer chromatographic separation. These differences are in accordance with the results of Suomi & Agranoff (1965) on the fatty acid composition of the double spot of spleen ceramide lactoside.

The alkali-stable lipids eluted from the Florisil column with chloroform–methanol (1:2, v/v) were chromatographed on a silicic acid column and eluted with chloroform–methanol (16:4, v/v). From a DEAE-cellulose column they were eluted in the non-acidic fraction. The hexose content of this glycolipid measured as galactose–glucose (molar ratio 2:1) accounted for 1·6% of the hexose of the total glycolipids. On thin-layer chromatography four spots were obtained that moved slower than the ceramide dihexosides and it is possible that they consist of two double spots. The slower-moving of them gave a positive reaction with ninhydrin. Further analysis of these glycolipids was not possible owing to lack of material; nevertheless, their higher polarity compared with that of ceramide dihexoside and their non-acidic character suggest that these compounds contain more than two hexose moieties/molecule.

Table 2. *Fatty acid composition of human leucocyte ceramide dihexoside fraction before and after separation by thin-layer chromatography*

The compositions were determined by gas–liquid chromatography. Values are expressed as percentages of total methyl esters.

Fatty acid	Total dihexoside	Upper spot	Lower spot
$C_{14:1}$	1·3	1·4	4·5
$C_{16:0}$	27·4	6·2	55·0
$C_{17:0}$	1·3	—	1·0
$C_{18:0}$	3·9	1·2	3·4
$C_{18:1}$	5·6	4·3	6·4
$C_{19:0}$	0·9	—	—
$C_{20:0}$	0·6	3·0	2·1
$C_{20:1}$	0·3	1·5	0·9
$C_{21:0}$	1·2	2·7	3·0
$C_{21:1}$	0·9	1·7	0·7
$C_{22:0}$	4·3	5·3	2·5
$C_{22:1}$	1·1	7·2	—
$C_{23:0}$	—	—	—
$C_{24:0}$	12·9	12·2	2·9
$C_{24:1}$	38·3	53·0	17·6

DISCUSSION

The isolated glycolipid that accounted for 15·2 and 16·4% of the total weight of the leucocyte lipid contained galactose and glucose in the molar ratio 2:1, and it was therefore a mixture of ceramide dihexosides. Partial acid hydrolysis indicated the presence of lactose, but the hexose ratio suggested that other disaccharides must also be present, like the galactosylgalactose found in ascites-sarcoma cells (Gray, 1965). Such a compound was not identified, however.

The carbohydrate composition of the major leucocyte glycolipid was similar to that of the ceramide dihexoside fraction isolated from tumour cells (Gray, 1965), since in both galactose was the predominant hexose. Nevertheless, it also resembled closely that of cytolipin H, the ceramide lactoside isolated from human epidermoid carcinoma (Rapport, Graf, & Yariv, 1961), and that of the ceramide dihexoside of erythrocytes and spleen (Yamakawa *et al.* 1962; Makita & Yamakawa, 1962). Ceramide dihexosides containing glucose and galactose in equal proportions have also been

isolated from human serum (Svennerholm & Svennerholm, 1963). The exceptional characteristic of leucocytes is that the ceramide dihexoside fraction comprised a high percentage of the total lipids. This may reflect a special role of this glycolipid in leucocyte structure and in their membrane function. The high content of ceramide dihexoside makes leucocytes unique among the tissues that have been shown to contain this glycolipid; therefore the presence of intact or destroyed leucocytes as contaminants in tissues analysed for glycolipids must be taken into consideration.

The isolated ceramide dihexosides were the major components of the extracted glycolipids. Another glycolipid fraction, presumably ceramide monohexoside, was present as a minor component. A similar pattern of glycolipid distribution has been reported for human spleen (Makita & Yamakawa, 1962), whereas the ceramide monohexoside content of rat tumour cells (Gray, 1965) was higher than that of the dihexoside. If this difference in the relative amounts of glycolipids is not due to species difference, it may reflect a functional or metabolic difference between normal and tumour cells. Gray (1963) found that different tumour cells contain relatively different quantities of glycolipids. Therefore the relative quantification of the glycolipids in leukaemic leucocytes may be useful in the study of the metabolism of leukaemic leucocytes.

The fatty acids of the leucocyte glycolipid, contrary to what has been reported for the ceramide dihexosides of spleen and tumour cells (Rapport, Skipski & Sweeley, 1961; Suomi & Agranoff, 1965; Gray, 1965), contain more $C_{24:0}$ acid. This is consistent with previous observations (Miras *et al.* 1965) according to which $C_{24:1}$ acids were synthesized *in vitro* from [^{14}C]acetate to much higher extent than $C_{24:0}$ acids. It is noteworthy that the leucocyte glycolipid did not contain either tricosanoic acid or α-hydroxytetracosanoic acid.

The leucocyte ceramide dihexoside will almost certainly possess haptenic activity (Rapport, 1960); it is therefore possible that this function is related to the immunological properties of the leucocytes, constituting part of their antigenic components. The more polar glycolipids, which under the experimental conditions used were isolated in very small quantities, have also been reported to possess blood-group activity (Hakomori & Jeanloz, 1961; Radin, 1957).

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The Metabolism of Fructose Polymers in Plants

TRANSFRUCTOSYLATION IN TUBERS OF *HELIANTHUS TUBEROSUS* L.

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1. A transfructosylase was separated from Jerusalem artichoke-tuber extracts. The partially purified enzyme was practically free from fructosans and its specific activity was increased more than sevenfold. 2. The enzyme was highly specific for terminal β-(2→1′)-linked fructofuranosyl residues. 3. The most active donor found was 1^F-fructosylsucrose, which had about five times the reactivity of the next higher homologues (degree of polymerization 4–6); (1-fructosyl)$_2$fructose was intermediate in reactivity; sucrose was inactive. 4. Sucrose, and polymers of high degree of polymerization (> 20), were the most efficient acceptors for transferred fructosyl residues of the compounds tested. 5. A variety of transferase activities appeared to be catalysed by the enzyme, namely, dismutation ('self-transfer') giving rise to a series of polymers ranging in degree of polymerization both above and below that of the original substrate, transfer to polymer giving rise to limited quantities of higher polymers, and transfer to sucrose; in the last case 1^F-fructosylsucrose/sucrose transfer (resulting in exchange labelling of sucrosyl groups) was used to investigate the mechanism of the reaction more fully. 6. Since fructose transfer can be demonstrated in the living plant tissue, the interrelationships of the various activities and their significance in fructosan metabolism are discussed.

In a previous paper (Edelman & Jefford, 1964) some properties of β-fructofuranosylases were reported, with regard to their involvement in mobilization of the fructosans of the Jerusalem artichoke tuber during sprouting. Characteristics of the compounds in the tuber have been described (Bacon & Edelman, 1950; Edelman & Jefford, 1964).

Several transfructosylase activities in this tissue have already been reported. They are summarized in equations (a)–(e), where Glc ~ Fru represents a sucrose residue, and the other D-fructofuranosyl units are linked β-(2→1′).

The relationships among these reactions were obscure, all the more so as some of them were investigated in ignorance of the existence of others, and enzyme preparations were likely to have contained fructosans of high degree of polymerization and hydrolases, all of which, in the light of later knowledge, were likely to take part in relevant reactions.

This paper is concerned with some detailed characteristics of transfructosylase in artichoke-tuber extracts with a view to clarification of the complexities of the reactions; the preparations used were free from contaminating fructosans and their hydrolases. A preliminary report of some of this work has been presented (Edelman & Dickerson, 1963).

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METHODS

Jerusalem artichokes were obtained from Chelsea Physic Garden and the Botanical Supply Unit, University of London.

Estimations. Total fructose (free and combined) was measured colorimetrically by the method of Cole (see Bacon & Bell, 1948), and reducing sugar by the method of Somogyi (1952) and Nelson (1944). Soluble protein was measured colorimetrically with Folin's reagents (see Lowry, Rosebrough, Farr & Randall, 1951), and spectrophotometrically by the nomograph method based on extinction coefficients of Warburg & Christian (1942).

Preparation of substrate-free enzyme. A column of DEAE-cellulose (Whatman DE50, powder form: 10g.; 2·5cm. × 18·0cm.) was washed with 10 bed-volumes of N-NaOH and then equilibrated with 5mM-phosphate buffer, pH6·35, at 5° until the pH of the effluent was 6·25. Artichoke juice (250ml.) prepared in the manner described below and containing 10ml. of 0·1M-cysteine and 0·1M-sodium diethyldithiocarbamate in phosphate buffer, pH7·0, was cooled to 5° and the precipitated inulin removed by centrifugation at 8000–10000*g* for 15min. The supernatant liquid (100ml.) was then applied to the column and washed through with

$$\text{Glc}\sim\text{Fru}+\text{Glc}\sim\text{Fru} \rightarrow \text{Glc}\sim\text{Fru-Fru}+\text{Glc} \quad \text{(a)}$$
(Dedonder, 1951*a*,*b*)

$$\text{Glc}\sim\text{Fru-(Fru)}_n+\text{Glc}\sim\text{Fru} \rightarrow \text{Glc}\sim\text{Fru-Fru}+\text{Glc}\sim\text{Fru-(Fru)}_{n-1} \quad \text{(b)}$$
(Edelman & Bacon, 1951*a*,*b*)

$$*\text{Glc}\sim*\text{Fru}+\text{Glc}\sim\text{Fru-Fru} \rightarrow *\text{Glc}\sim*\text{Fru-Fru}+\text{Glc}\sim\text{Fru} \quad \text{(c)}$$
(Edelman & Recaldin, 1961)

$$2n\text{Glc}\sim\text{Fru-Fru} \rightarrow \text{Glc}\sim\text{Fru-(Fru)}_{2n}+(2n-1)\text{Glc}\sim\text{Fru} \quad \text{(d)}$$
(Recaldin, 1961)

$$n\text{Glc}\sim\text{Fru-(Fru)}_n \rightarrow \text{Glc}\sim\text{Fru}+\text{Glc}\sim\text{Fru-Fru}+\text{Glc}\sim\text{Fru-(Fru)}_2+\ \ldots\ \text{etc.} \quad \text{(e)}$$
(Dickerson, 1965)

the same buffer at 50–60 ml./hr. until the effluent contained $<3{\cdot}0\,\mu$g. of total fructose/ml. The column was then eluted after the method of Bock & Ling (1954) with a linear gradient of ionic strength (increasing from 0·02 to 0·6) with NaCl and phosphate buffer (Na_2HPO_4 and KH_2PO_4), pH 6·1. The eluate was collected at approx. 1 ml./min. in 40 ml. fractions. The fractions were assayed for transferase activity (μmoles of fructose transferred/ml. of incubated system/hr./μg. of protein) by incubating dialysed samples containing equal amounts of protein (0·5 μg.) with 25 mM-[U-^{14}C]-1^F-fructosylsucrose in 0·02 M-phosphate buffer, pH 6·35, for 1 hr. at 25°. The products of the incubation were separated by chromatography and their radioactive content was determined. Fractions containing the activity which emerged from the column between the ionic strengths 0·29 and 0·45 were bulked and subjected to ultrafiltration in $\frac{1}{4}$ in. diam. Visking tubing and washing with 5 mM-phosphate buffer, pH 6·1, at 5°. The residual solution was freeze-dried to yield approx. 100 mg. of pale-brown powder, which was stored at −20°. When dissolved in water this preparation gave pH 6·1; it contained no detectable fructose and had a specific enzyme activity 7·5 times that of the original protein in the juice. It is henceforth called 'buffered transferase'. No hydrolase activity was detected in this preparation (see also the Results section) because: (1) its activity in tuber juice is much lower than that of the transferase; (2) it was presumably at least partially separated on the column; (3) conditions of pH were unsuitable (Edelman & Jefford, 1964).

Enzymic procedure. Incubations were carried out at 25°, pH 6·1. The recommendations of the International Union of Biochemistry were followed for general procedure. Small volumes of incubation mixtures ($<50\,\mu$l.) were contained in 20 mm. × 3 mm. (internal diam.) glass tubes; larger volumes (50–150 μl.) were placed in specially designed tubes (with a constricted neck to reduce evaporation) with an internal volume 150 μl. Pipetting of small volumes (1–20 μl.) was in E-Mil Gold-Line auto zero high-precision pipettes or Drummond Microcaps (Drummond Scientific Co., Philadelphia, Pa., U.S.A.). Enzyme activity was terminated by the use of 10 mM-$HgCl_2$; 20–30 μl. of this solution was applied to the chromatogram before the sample.

Preparation of enzyme-free substrates. Tuber juice contains a series of fructosans ranging from sucrose to inulin (Edelman & Jefford, 1964). One litre of juice was expressed from the tissues of debudded and washed tubers macerated in a Waring Blendor, and the mixed oligo- and poly-saccharides were precipitated at 0° with ethanol (final concn. 95%, v/v); approx. 30 g. of mixed fructosans was obtained after separation by centrifugation at 0° for 15 min. at 10000*g*.

Partial separation of the oligosaccharides from the polysaccharides was effected both by differential precipitation in 70–75% ethanol, whence most of the oligosaccharide fraction (D.P.* $\leqslant 6$) remained in solution, and by the use of column chromatography. For the latter, the method of Jones, Wall & Pittet (1960) was followed with slight modifications: Dowex 50 W (200–400 mesh; 2% divinylbenzene) in the Li^+ form was carefully packed in a 200 cm. × 1·7 cm. column and the polymer mixture containing about 0·5 g. of total fructose in 5·0 ml. was applied and the column eluted with water at about 15–20 ml./hr.; individual oligosaccharides were not completely separated but 10 ml. fractions were collected in which 50–70% of total fructose was in one compound within the lower D.P. range (3–10); the compounds left the column in descending order of molecular weight. The crude fractions were concentrated under an infrared lamp in a current of air, applied to Whatman 3MM paper and the individual fructosans separated by descending chromatography at 25° in propan-1-ol–ethyl acetate–water [for 48 hr. in 7:1:2 (by vol.) for monosaccharides, up to 5 days in 6:1:3 (by vol.) for oligosaccharides to about D.P. 14, and for up to 3 weeks in 11:2:7 (by vol.) for higher polymers and 'inulin' (> D.P. 25)]. Identification of the compounds on chromatograms was carried out with sprays used by Edelman & Jefford (1964), after which the polymers were eluted from the paper with water. Isolated polymers were further purified by rechromatography.

O-β-D-Fructofuranosyl-(2→1′)-D-fructose (1-fructosylfructose) and *O*-β-D-fructofuranosyl-(2→1′)-*O*-β-D-fructofuranosyl-(2→1′)-D-fructose [(1-fructosyl)$_2$fructose] were obtained by the partial hydrolysis of inulin with 0·1 N-oxalic acid at 60° for 30 min. and were separated by paper chromatography in propan-1-ol–ethyl acetate–water (7:1:2, by vol.) for 40 hr.

Preparation of radioactive substrates. A detached leaf of *Canna indica* was exposed in sunlight to $^{14}CO_2$ prepared by the action of 50% (v/v) lactic acid on $Ba^{14}CO_3$ (125 μc/mg.) obtained from The Radiochemical Centre, Amersham, Bucks.; [U-^{14}C]sucrose (100 μc/mg.) isolated from the leaf was incubated with a Taka-diastase preparation (1%, w/v; Parke Davis and Co. Ltd., Hounslow, Middlesex) for 24 hr. at 30°. The [U-^{14}C]-*O*-β-D-fructofuranosyl-(2→1′)-*O*-β-D-fructofuranosyl-(2→←1′)-α-D-glucopyranoside (1^F-fructo-

* Abbreviation: D.P., degree of polymerization.

sylsucrose) isolated from the digest by chromatography as above is known to be contaminated with *O*-β-D-fructofuranosyl-(2→6')-*O*-α-D-glucopyranosyl-(1→←2)-β-D-fructofuranoside (6^G-fructosylsucrose), which runs together with the 1^F compound on the chromatogram (Bacon, 1960 *a*,*b*). Purer radioactive substrates were therefore prepared as follows: 0·40 mg. (100 μc) of repurified [U-^{14}C]sucrose was incubated with 0·52 mg. of carrier sucrose and 1·27 mg. of repurified 1^F-fructosylsucrose in 50 μl. of solution (i.e. both substrates at 0·05 M), containing 0·50 mg. of the buffered transferase, for 15 min. at 25°. The products of the incubation were isolated by chromatography as above. The 1^F-fructosylsucrose/sucrose-transferase activity equilibrated the radioactivity and gave rise to about 45 μc of 1^F-fructosylsucrose labelled only in the sucrose residue; a small amount (about 6% of the total radioactivity) of the next higher homologue [(1^F-fructosyl)$_2$sucrose] was also formed. Approx. 300 μg. (10–12 μc) of 1^F-fructosylsucrose thus isolated was incubated alone in 10 μl. of water (0·06 M) with 0·26 mg. of the buffered transferase for 2 hr. at 25°. From this incubation it was possible to isolate, in addition to 1^F-fructosylsucrose, the higher homologues (1^F-fructosyl)$_2$sucrose, (1^F-fructosyl)$_3$sucrose and (1^F-fructosyl)$_4$-sucrose formed by transfructosylation and all labelled in the sucrose residue alone. After repurification by chromatography the final yields were approx. 1 mg. containing 40 μc, 0·26 mg. containing 8·5 μc, 0·06 mg. containing 1·5 μc and 0·04 mg. containing 0·75 μc for the polymers of D.P. 3, 4, 5 and 6 respectively.

Detection and estimation of radioactivity. Radioactive areas on paper chromatograms were located with a thin end-window Geiger–Müller counter (Monitor Radiation Alpha Beta, McMichael Radio Ltd.; and a portable Airmec type 255) or by exposure to Kodirex No-screen X-ray film. Quantitative estimation of ^{14}C was achieved either by chromatogram scanning (Isotope Developments Ltd. scanner 2029 with scaler 1700), or by elution of the compounds followed by thin end-window counting on duralumin disks, or by direct estimation on the radioactive areas cut from the paper and put through an automatic sample changer (I.D.L. 2014 fitted with 2 in. Geiger–Muller tube 2B2; average of both sides taken). Estimates based on these three methods agreed closely.

RESULTS

Self-transfer. This term is used when a compound acts both as fructosyl donor and acceptor. Self-transfer of the trisaccharide 1^F-fructosylsucrose is shown, on a molar basis, in Fig. 1(*a*); all fructose was accounted for at all stages in the reaction by

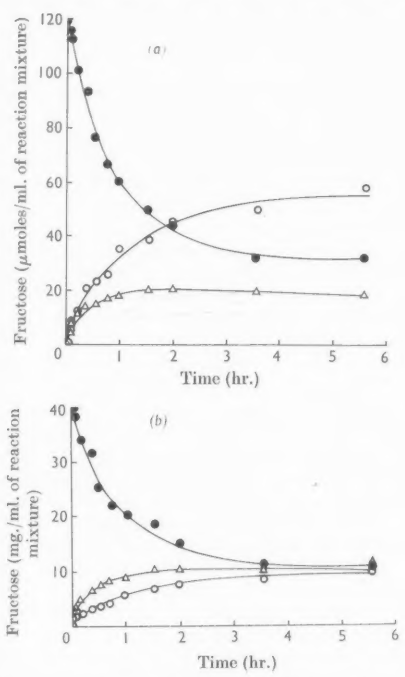

Fig. 1. Action of the transferase upon 1^F-fructosylsucrose. The incubation mixture contained 0·20 M-1^F-fructosylsucrose and 2·5 mg. of buffered transferase/ml. Results were calculated from analyses of total fructose content (*a*) as μmoles/ml. and (*b*) as mg./ml. ○, Sucrose; ●, D.P. 3; △, D.P. 4.

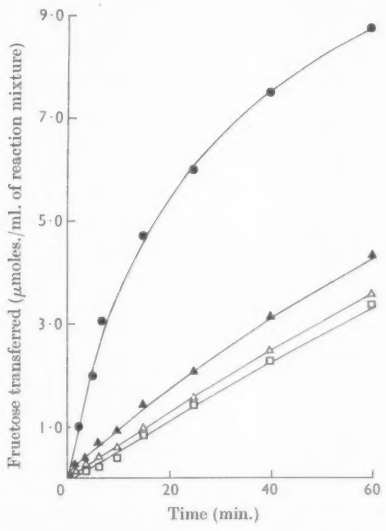

Fig. 2. Rate of transfer of fructose from individual oligosaccharides in the presence of transferase. Each incubation mixture contained 0·03 M-substrate labelled with ^{14}C in the sucrosyl group and 2·6 mg. of buffered transferase/ml. Results were calculated from assays of radioactivity. ●, D.P. 3; △, D.P. 4; ▲, D.P. 5; □, D.P. 6.

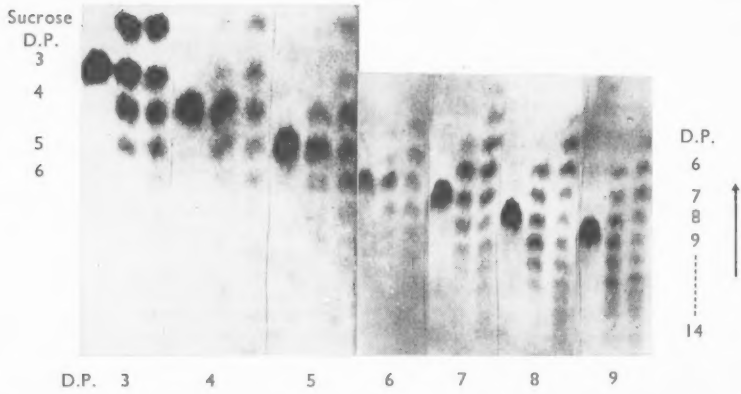

Fig. 3. Disproportionation of individual oligosaccharides by transferase. Mixtures contained the appropriate substrate (0·05 M) as given at the bottom of the Figure and 3·0 mg. of buffered transferase/ml. The three samples shown here from left to right for each substrate were removed for paper chromatography after 0, 1 and 5 hr. incubation respectively. Chromatograms were sprayed with benzidine–trichloroacetic acid (Edelman & Bacon, 1951*a*,*b*). Direction of development is shown by the arrow. All numerals refer to D.P. values.

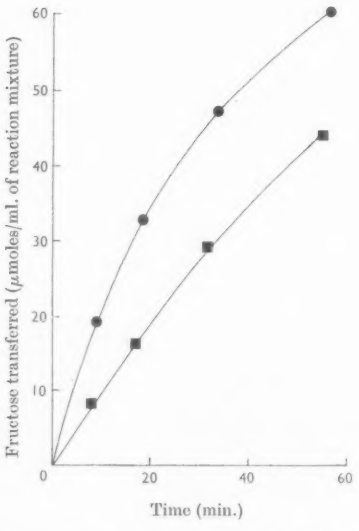

Fig. 4. Comparison of rates of self-transfer with 1^F-fructosylsucrose or (1-fructosyl)$_2$fructose in the presence of transferase. Each incubation mixture contained 0·24 M-1^F-fructosylsucrose (●) or 0·28 M-(1-fructosyl)$_2$fructose (■), and 2·5 mg. of buffered transferase/ml. Results were calculated from analyses of total fructose content.

the oligosaccharides present in the reaction mixture: the major components are shown, but some of higher D.P. were present in small amounts which together had a fructose content that was 15% of the total present in the reaction mixture. Fig. 1(*b*) shows that at equilibrium the major reaction products contain equal weights of fructose, a phenomenon which can be observed among the oligo- and poly-saccharides found in the juice of dormant tubers. Similar activity was shown with the tetra-, penta- and hexa-saccharides. The rate of reaction with these substances is shown in Fig. 2, which demonstrates the greater rate of transfer with the trisaccharide as substrate. Incubation of a variety of oligosaccharides individually leads to the situation demonstrated in Fig. 3. The absence of free fructose in these experiments shows that the buffered transferase is free from hydrolytic fructosylases. Investigation of the specificity of the enzyme showed that terminal β-(2→1′)-linked fructofuranosyl groups were transferred, as the enzyme was active against all the natural oligosaccharides tried (D.P. 3–9), as well as against (1-fructosyl)$_2$fructose (Fig. 4). The enzyme was inactive against 1-fructosylfructose, sucrose, maltose, melibiose, melezitose, raffinose, 6^G-fructosylsucrose and 6^F-fructosylsucrose. The effect of varying substrate concentration is shown in Fig. 5. Because of the nature of the reaction both donor

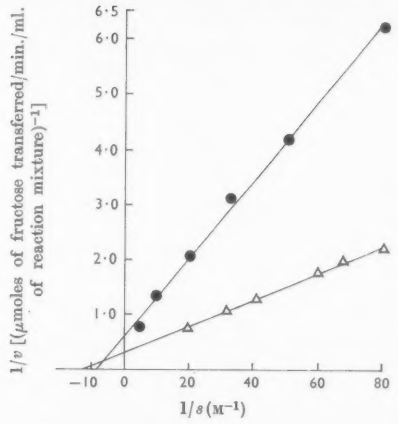

Fig. 5. Lineweaver–Burk plots for self-transfer. Each incubation mixture contained substrate labelled with ^{14}C in the sucrose residue; those with 1^F-fructosylsucrose (●) contained 2·0 mg. of buffered enzyme/ml. and were incubated for 5 min.; those with $(1^F\text{-fructosyl})_2$sucrose (△) contained 3·65 mg. of enzyme/ml. and were incubated for 15 min. Results were calculated from assays of radioactivity.

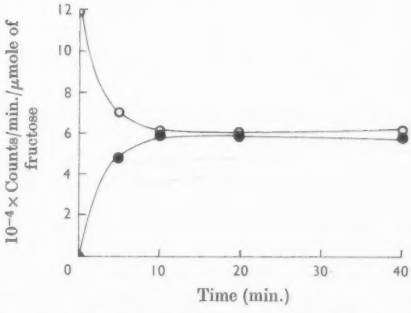

Fig. 6. Effect of the transferase upon an equimolar mixture of 1^F-fructosylsucrose and [U-^{14}C]sucrose. The incubation mixture contained each substrate at 25 mM concentration and 5·4 mg. of buffered transferase. Results were calculated from assays of radioactivity; ○, sucrose; ●, 1^F-fructosylsucrose.

and acceptor concentrations necessarily varied together, but the apparent K_m values were calculated to be 0·125 M (D.P. 3) and 0·08 M (D.P. 4).

Transfer to sucrose. (1) Trisaccharide as fructosyl donor. Reaction (c) above is demonstrated by the action of the buffered transferase on an equimolar mixture of 1^F-fructosylsucrose and [U-^{14}C]sucrose

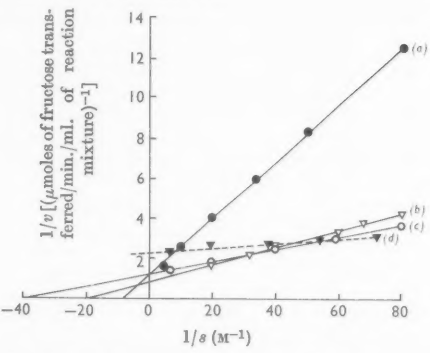

Fig. 7. Effect of reactant concentration on the 1^F-fructosylsucrose/sucrose transfer reaction. Mixtures were incubated for 4 min. and contained a known weight (about 1 mg.) of buffered transferase/ml., and substrates as follows: ●, 1^F-fructosyl[U-^{14}C]sucrose alone; ▽, equimolar 1^F-fructosylsucrose and [U-^{14}C]sucrose; ○, varying 1^F-fructosylsucrose and 25 mM-[U-^{14}C]sucrose; ▼, varying [U-^{14}C]-sucrose and 25 mM-1^F-fructosylsucrose. Results were calculated from assays of radioactivity and for 1·0 mg. of transferase preparation; e.g., where sucrose is acceptor, the appearance of label in the trisaccharide was measured and was found to be equivalent to its loss from the sucrose, and hence fructose transfer was calculated on the basis of equation (c).

(Fig. 6). The pH optimum was found to be a plateau between 6·0 and 7·0 with a sharp fall on either side.

The effect of varying the concentrations of the reactants is shown in Fig. 7: initial specific activities were known, and subsequent relative ^{14}C contents of all reaction products were measured; fructose transfer was calculated on the basis of proportionality of migration of label. Under the conditions of these experiments (i.e. < 10% transfer of fructose residues from the original substrate) we have found by measurement of both ^{14}C migration and fructose content that no detectable self-transfer occurs when sucrose is present. Significant self-transfer was found only after the equilibration reaction had reached completion.

Specificity: a similar reaction was observed with $(1\text{-fructosyl})_2$fructose used as donor; this reaction proceeded at one-quarter of the rate of that with 1^F-fructosylsucrose as donor. Raffinose behaved like sucrose as an acceptor, and 1^F-fructosylraffinose in the same way as the sucrose analogue as a fructosyl donor; in both cases the reaction rate was the same as for 1^F-fructosylsucrose/sucrose transfer.

(2) Oligo- and poly-saccharide as donor. The specificity of the buffered transferase towards individual compounds of the natural series is given in

Table 1. *Transfer of fructose residues from oligosaccharides to sucrose*

(*A*) Mixtures contained 25mM-donor, 25mM-[U-^{14}C]-sucrose, 2·4mg. of buffered transferase/ml., and were incubated for 10min. Results were calculated from assays of radioactivity. (*B*) As (*A*) but mixtures contained 5mM-donor (except where stated otherwise) and 3·2mg. of enzyme/ml., and were incubated for 15min.

	Fructose transferred (μmoles/hr./ml. of reaction mixture)	
D.P. of donor	(*A*)	(*B*)
		(25mM) 33·8
3	46·7	(5mM) 14·6
4	2·34	
5	3·71	
6	3·09	1·92
7	8·4	
8	17·4	7·1
9	16·2	
12		7·6
15		5·8
18		4·4
20		5·5
24–26		5·1
27–35		6·3

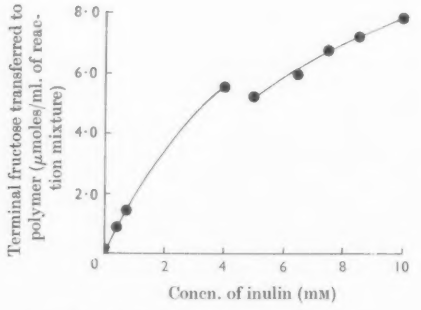

Fig. 8. Effect of concentration of inulin primer upon fructose transfer. Mixtures contained inulin (D.P. ⩾25) (average mol.wt. taken as 5000), 25mM-[U-^{14}C]-1^{F}-fructosylsucrose, and 4mg. of buffered transferase/ml., and were incubated for 2hr. Results were calculated from assays of radioactivity. The two curves denote different occasions.

Table 1; all the compounds tested were poorer donors than the trisaccharide, particularly the lower members (D.P. 4–6).

Transfer to polymer. Addition of [U-^{14}C]-1^{F}-fructosylsucrose to artichoke juice indicated that

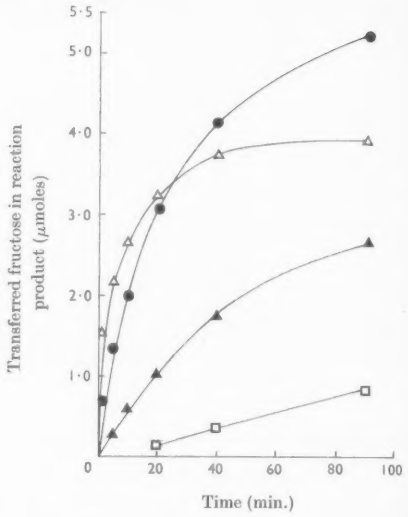

Fig. 9. Course of fructose transfer from 1^{F}-fructosylsucrose in the presence of inulin. Conditions were similar to those given for Fig. 7, except that concentration of primer was 5mM and that of the buffered transferase was 3·2mg./ml. Results are plotted as the amount of transferred fructose appearing in: △, D.P. 4; ▲, D.P. 5; □, D.P. 6; ●, D.P. >20.

radioactive material was transferred preferentially to the polysaccharide, as shown by paper chromatography, namely to material of R_F zero. Graded acid hydrolysis of eluted polysaccharide gave a series of ^{14}C-labelled oligofructosides showing that the transferred fructose had become an integral part of the polysaccharide molecules. Fig. 8 shows the effect of concentration of priming polymer upon the action of purified enzyme. During this reaction self-transfer of the trisaccharide also occurred, giving rise to higher oligosaccharides; a time-course of the total transfructosylation occurring in a similar experiment is shown in Fig. 9. This Figure demonstrates preferential transfer to material of high D.P. as the D.P. >20 curve is steep although the acceptor concentration of polymer (about 5mM) was considerably lower than that of the trisaccharide (25mM), which gave rise to the D.P. 4–6 fractions.

Investigation of the acceptor ability of several of the intermediate oligosaccharides gave results shown in Table 2, which demonstrates that from D.P. 6 upwards the accepting ability is fairly constant and is considerably greater than that of the trisaccharide (self-transfer).

Table 2. *Acceptor ability of members of the inulin series*

Mixtures contained 5mM-primer, 2·5mM-[U-^{14}C]-1^{F}-fructosylsucrose and 2·9mg. of buffered transferase/ml., and were incubated for 15min.

Primer... ...	None*	D.P. 6	D.P. 12	D.P. 18	D.P. 21	D.P. 27–*n*
Fructose transferred (μmoles/hr./ml. of reaction mixture)						
to form D.P. 4	12·8	9·7	9·6	8·6	8·9	8·7
to form D.P. 5†	3·9	2·2	2·1	1·5	1·9	1·5
to the primer	—	11·5 (10·1)‡	10·0 (9·5)‡	7·4 (8·7)‡	8·4 (9·0)‡	10·9 (10·7)‡
Total fructose transferred (%)	16·7	23·4	21·7	17·5	19·2	21·1

* Control.

† Radioautography indicated that reactions initiated by self-transfer of D.P. 3 proceeded no further than production of material of D.P. 5.

‡ Values adjusted for equal total transfer (average 20·6).

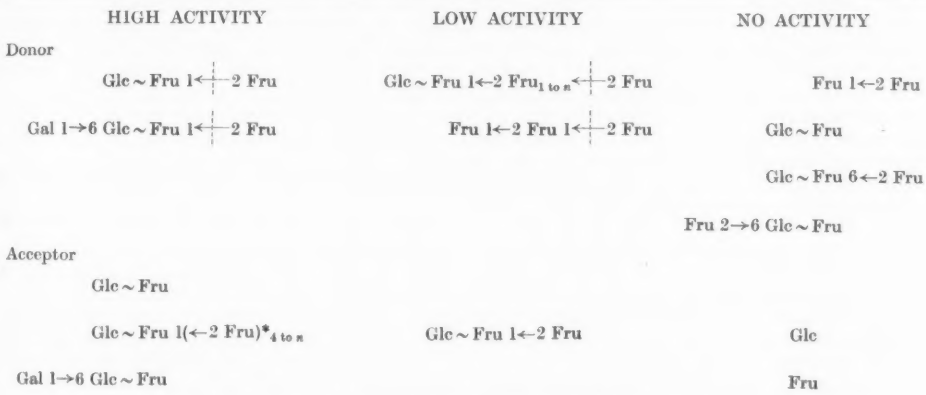

Fig. 10. Donor and acceptor specificity of the artichoke transferase.

* The tetra- and penta-saccharides were not investigated as the products of the 1^{F}-fructosylsucrose self-transfer reaction are identical with those of direct transfer from 1^{F}-fructosylsucrose to the two higher oligosaccharides; the highest value of *n* was not established but was at least 23.

DISCUSSION

With the buffered transferase described here, which was partially purified and substantially free from fructosans and hydrolases acting upon fructosans, the specificity of the transfructosylase for the most relevant sugars is summarized in Fig. 10; these activities could not be ascribed to separate enzymes, hence reactions (b)–(e) are considered to be due to the same enzyme acting under the appropriate conditions. The course of the reactions is conditioned by the nature and concentrations of the donor and acceptor substrates: starting even from a single substance the situation rapidly becomes highly complex as the reaction products themselves act as fructose donors and acceptors which may differ from one another and from the original substrate in their reaction characteristics. Thus sucrose appears more rapidly the lower the D.P. of the donor oligosaccharide, and, as sucrose is a preferential acceptor, disproportionation is substantially suppressed. For a similar reason the presence of compounds of D.P. greater than about 6 appears to suppress transfer among the lower members.

Since the presence of sucrose in a reaction mixture with the trisaccharide as the sole donor substrate suppresses self-transfer, a competition exists for transferred residues according to the equations (f) and (g).

A comparison of curves (*a*), (*b*) and (*c*) in Fig. 7 shows that the introduction of sucrose, i.e. the substitution of 1^{F}-fructosylsucrose by sucrose as acceptor, decreases the K_m for transfer of fructose residues from 1^{F}-fructosylsucrose. The effect might be due to the formation of an enzyme–acceptor

$$\text{Glc}\sim\text{Fru-Fru} + {}^{*}\text{Glc}\sim{}^{*}\text{Fru} \rightarrow \text{Glc}\sim\text{Fru} + {}^{*}\text{Glc}\sim{}^{*}\text{Fru-Fru} \quad \text{(f)}$$

$$2\ \text{Glc}\sim\text{Fru-Fru} \rightarrow \text{Glc}\sim\text{Fru} + \text{Glc}\sim\text{Fru-Fru-Fru} \quad \text{(g)}$$

complex containing sucrose, which imposes a low K_m on the donating site for trisaccharide, as compared with an enzyme–acceptor complex containing trisaccharide, which imposes a high K_m on the donating site for trisaccharide. Alternatively, the affinity of the donating site for trisaccharide may be relatively high, the rate-limiting step being conditioned by the affinity of the enzyme for acceptor (Stringer & Tsuchiya, 1958), this affinity being higher for sucrose than for trisaccharide.

In the 1^F-fructosylsucrose self-transfer reaction (Fig. 7, curve *a*) the concentration of the acceptor necessarily varies with the donor; hence the reaction denoted by curve (*b*) is more comparable with the 1^F-fructosylsucrose self-transfer reaction than that of curve (*c*), since the concentrations of both donor and acceptor, namely 1^F-fructosylsucrose and sucrose, were varied together and were equimolar throughout; the differences, however, between the slope of curves (*b*) and (*c*) may be considered only marginal, and it would appear from the nearly horizontal slope of curve (*d*) (varying sucrose and constant 1^F-fructosylsucrose) that changing the sucrose concentration over the molarity range chosen makes little difference to the overall rate of reaction. Nevertheless, introduction of sucrose (at concentrations between 12·5 and 150mM) into the reaction mixture does suppress self-transfer and also increases the rate of fructose turnover (except at relatively high 1^F-fructosylsucrose concentration), and although over the fairly wide range of concentrations examined here sucrose appears to be equally effective in enhancing the rate of reaction, this does not mean that the reaction is independent of sucrose concentration at lower values.

Significance of transfructosylation in the living tissue. Edelman, Recaldin & Dickerson (1963) have already shown that reaction (c) above occurs in the living tissue, both in tubers attached to the plant and in incubated tuber slices. The distribution of fructose among the various compounds in the tuber follows a particular pattern, namely that the total amount present in each of the compounds tends to equality (see Jefford & Edelman, 1961). The transfructosylase described in the present work effects a similar result in cell-free extracts (see Figs. 1 and 10) and in view of the predicted result that labelled sucrose residues would progressively appear in higher oligosaccharides in the living tissue, this enzyme is demonstrably the operative factor.

Whether this enzyme is involved in polymer synthesis *in vivo* still remains in doubt. Certainly, in the presence of added primer, it can form long chains with the trisaccharide as donor, but the ratio of synthetic polymer to added primer was very low ($< 1:25$, w/w); moreover, translocated sucrose is the primary substrate in the tuber (Edelman & Popov, 1962; Dickerson & Edelman, 1966) and the enzyme does not convert sucrose into trisaccharide. The system of polymer synthesis from sucrose must be more complicated than a straightforward transfructosylation, as extensive conversion of glucosyl into fructosyl residues occurs.

The functional significance of the transfructosylase therefore may lie in controlling the concentrations of sucrose and higher polymers in the tissue [see also the discussion by Edelman & Jefford (1964) of the role of hydrolases in this respect].

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The Route of Ethanol Formation in *Zymomonas mobilis*

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1. Enzymic evidence supporting the operation of the Entner–Doudoroff pathway in the anaerobic conversion of glucose into ethanol and carbon dioxide by *Zymomonas mobilis* is presented. 2. Cell extracts catalysed the formation of equimolar amounts of pyruvate and glyceraldehyde 3-phosphate from 6-phosphogluconate. Evidence that 3-deoxy-2-oxo-6-phosphogluconate is an intermediate in this conversion was obtained. 3. Cell extracts of the organism contained the following enzymes: glucose 6-phosphate dehydrogenase (active with NAD and NADP), ethanol dehydrogenase (active with NAD), glyceraldehyde 3-phosphate dehydrogenase (active with NAD), hexokinase, gluconokinase, glucose dehydrogenase and pyruvate decarboxylase. Extracts also catalysed the overall conversion of glycerate 3-phosphate into pyruvate in the presence of ADP. 4. Gluconate dehydrogenase, fructose 1,6-diphosphate aldolase and NAD–NADP transhydrogenase were not detected. 5. It is suggested that NAD is the physiological electron carrier in the balanced oxidation–reduction involved in ethanol formation.

Considerable significance has been attached to *Zymomonas mobilis* (*Pseudomonas lindneri*) since it is cited as a model micro-organism for the anaerobic Entner & Doudoroff (1952) pathway of degradation of glucose (see, e.g., Gunsalus & Shuster, 1961; Wood, 1961). Evidence to support such claims has hitherto been based almost solely on isotopic data, and no enzymic details have been presented. It was the purpose of our investigation to confirm the assumptions of previous workers by demonstrating the presence of the relevant enzymes in this micro-organism, and particularly to examine the coenzyme specificities of the reactions involved in ethanol formation. Much of the work reported was carried out during 1960–61 and a preliminary account was communicated to The Biochemical Society at that time (Ribbons & Dawes, 1961).

Kluyver & Hoppenbrouwers (1931) showed that *Z. mobilis* ferments glucose to approximately 1·8moles each of ethanol and carbon dioxide, and 0·2mole of lactate/mole of hexose utilized. Gibbs & DeMoss (1951, 1954) and DeMoss (1953) confirmed the fermentation balance when washed cell suspensions were allowed to ferment glucose or fructose in the absence of a nitrogen source. Using specifically labelled [^{14}C]glucose they showed that the carbon dioxide arose from C-1 and C-4 of the glucose molecule. [2-^{14}C]Glucose was fermented to yield ethanol labelled in the carbinol carbon atom. Earlier, DeMoss & Gibbs (1952) reported the presence of pyruvate decarboxylase ('carboxylase'), glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and fructose 1,6-diphosphate aldolase in cell extracts.

From the results of a radio-respirometric study Stern, Wang & Gilmour (1960) concluded that *Z. mobilis* ferments glucose by an Entner–Doudoroff pathway. A molar-growth-yield study by Bauchop & Elsden (1960) is also consistent with this pathway, as these workers were able to demonstrate that the energy yield per mole of hexose was one-half of the corresponding value for *Saccharomyces cerevisiae*. We have confirmed this finding (Ribbons, Dawes & Rees, 1962) and details are presented in the next paper (Dawes, Ribbons & Rees, 1966). Since our work was completed a paper on the growth yield and growth characteristics of *Z. mobilis* has appeared (Belaïch & Senez, 1965).

EXPERIMENTAL

Materials

Organism. The strain of *Z. mobilis* used was N.C.I.B. 8938. This strain is no longer motile, an observation confirmed by Dr J. L. Shimwell.

Media and growth of the organism. Z. mobilis was maintained initially as stab-cultures in nutrient agar containing glucose (2%, w/v). Slants of the same media were used

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subsequently when we found that *Z. mobilis* could tolerate and consume oxygen. Liquid cultures were grown at 30° in Erlenmeyer flasks containing (g./100ml. of water): glucose, 2; Bacto yeast extract (Difco), 1; Bacto-Peptone (Difco), 1; pH 6·8. Bulk cultures (8l.) were grown in 10l. flat-bottomed flasks and were inoculated with 10ml. of an actively gassing culture. Small volumes (up to 450ml.) were harvested by centrifuging at 18000*g* for 5min. Larger (8l.) cultures were harvested at 55000*g* on a Sharples no. 1A laboratory Super-Centrifuge driven by compressed air. Cells were washed twice with either phosphate buffer (pH 7·0) or water, and then either used immediately or, alternatively, stored at −18° as a paste until required.

Cell extracts. These were prepared by submitting cell suspensions (approx. 5g. wet wt./25ml. of distilled water) to ultrasonic oscillation in either a Raytheon 9kcyc./sec. ultrasonic oscillator for 25–30min., or an MSE ultrasonic disintegrator for 2min.; cooling was achieved by running tap water (9–15°) or by crushed ice respectively. The resultant suspension was centrifuged at approx. 18000*g* for 30min. Some extracts were further clarified in the Spinco (model L) preparative ultracentrifuge at 97000*g* or higher for 2hr. at 0°.

Enzymes. Crystalline muscle aldolase was obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany.

Chemicals. NAD, NADP, $NADH_2$ and $NADPH_2$ were obtained from C. F. Boehringer und Soehne G.m.b.H., and from Sigma Chemical Co., St Louis, Mo., U.S.A. Triose phosphates were obtained as a solution of the esters (TEP solution) from C. F. Boehringer und Soehne G.m.b.H. 6-Phosphogluconate and glucose 6-phosphate were obtained as barium and sodium salts from Sigma Chemical Co. and from British Drug Houses Ltd., Poole, Dorset. The monobarium salt of 3-deoxy-2-oxo-6-phosphogluconate was a generous gift from Dr W. A. Wood. Other reagents were of analytical grade wherever possible.

Buffer solutions. Phosphate buffer was 0·067M-KH_2PO_4 adjusted to the required pH with 5N-NaOH; glycylglycine buffer of the appropriate concentration was adjusted to the required pH with 5N-NaOH. Pyrophosphate buffer was 0·1M-sodium pyrophosphate, pH 8·5. Other buffers (see Table 3) were prepared according to Gomori (1955).

Analytical methods

Chemical estimations. Glucose was determined by the method of Nelson (1944). Ethanol was estimated by dichromate oxidation of a neutral steam-distillate, followed by back-titration of the excess of 0·04N-$K_2Cr_2O_7$ with KI and 0·05N-$Na_2S_2O_3$ as described by Neish (1952). Lactate was estimated by the procedures of Hullin & Noble (1953) and Barker & Summerson (1941). Pyruvate was determined by the extraction procedure of Friedemann & Haugen (1943). Total keto acids were determined by the semicarbazide method of MacGee & Doudoroff (1954). Oxygen consumption and CO_2 evolution were measured in constant-volume Warburg respirometers at 30°. Evolution of CO_2 was always estimated with a gas phase of N_2+CO_2 (95:5) (Umbreit, Burris & Stauffer, 1957). Spectrophotometric determinations were made with Hilger Uvispek, Unicam SP.500 and SP.600 instruments and quartz cells with 1cm. light-path. Inorganic phosphate was determined by the method of Fiske & Subbarow (1925). Alkali-labile phosphate was determined as described by Umbreit *et al.* (1957).

3-Deoxy-2-oxo-6-phosphogluconate was determined either by the semicarbazide method of MacGee & Doudoroff (1954) or by periodate oxidation followed by thiobarbituric acid treatment as described by Srinivasan & Sprinson (1959). The phosphorylated form of 3-deoxy-2-oxogluconate is not readily oxidized by periodate (MacGee & Doudoroff, 1954) and maximum colour formation required a 60min. reaction period. Even then, as observed by Meloche & Wood (1964), the colour yield was much less than that obtained with the unphosphorylated compound. We found the molar extinction coefficient under these conditions to be 1500 at 548mμ; that of the dephosphorylated compound is 44000 (Weissbach & Hurwitz, 1959). Hydrazine interferes with this reaction and was omitted from the assay mixture. Protein was estimated by the biuret method of Stickland (1951).

Chromatography. Descending paper chromatograms were developed in propan-1-ol–formic acid (90%, w/v)–water (6:3:1, by vol.) (De Ley, 1955). After drying at room temperature phosphate esters were located by dipping in the reagent of Burrows, Grylls & Harrison (1952) followed by drying at room temperature and irradiation with a u.v. lamp.

Enzyme assays. The assay methods for glucose 6-phosphate dehydrogenase, ethanol dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase, $NADH_2$ oxidase, NAD–NADP transhydrogenase and the combined activities of 6-phosphogluconate dehydratase and 3-deoxy-2-oxo-6-phosphogluconate aldolase are given in the legends to the appropriate Figures.

Glucose 6-phosphate dehydrogenase in crude extracts was also assayed manometrically by O_2 consumption. Each flask contained: 20μmoles of glucose 6-phosphate, 0·5ml.; 0·3M-glycylglycine buffer, pH 7·3, 1·0ml.; 2mM-NAD, 1·0ml.; water, 0·3ml.; cell extract, 0·2ml. The centre well contained 0·2ml. of 20% (w/v) KOH. Substrate was omitted from the control.

Glyceraldehyde 3-phosphate dehydrogenase was also assayed manometrically. Each flask contained: 0·067M-phosphate buffer, pH 7·1, 1·0ml.; 0·1M-glutathione, 0·2ml.; 20mM-ADP, 0·5ml.; 2mM-NAD, 0·2ml.; triose phosphate ester solution (approx. 16mM), 0·5ml.; 0·08M-$MgCl_2$, 0·1ml.; cell extract, 0·5ml. The centre well contained 0·2ml. of 20% (w/v) KOH. Substrate was omitted from the control. Glucose dehydrogenase and gluconate dehydrogenase were both assayed by the reduction of 2,6-dichlorophenol-indophenol in tubes of the type described by Peel (1960). The assay system contained: 0·2M-glycylglycine, pH 7·3, 2·0ml.; 0·05% (w/v) 2,6-dichlorophenol-indophenol, 0·2ml.; 20mM-substrate, 1·0ml.; water, 1·5ml.; cell extract, 0·3ml. The change in extinction at 600mμ was measured over a period of 5min.

Hexokinase was demonstrated spectrophotometrically by measuring the reduction of NAD by the glucose 6-phosphate produced by phosphorylation of glucose. The system contained: 0·08M-$NaHCO_3$, 1·0ml.; 0·4M-$MgCl_2$, 0·1ml.; 10mM-glucose, 0·5ml.; 50mM-ATP, 0·3ml.; 0·01M-tris, pH 7·0, 0·5ml.; cell extract, 0·1ml.; 2mM-NAD (or NADP), 0·1ml. The NAD was added last and the change in E_{340} measured. Gluconokinase was demonstrated spectrophotometrically in the following system: 0·1M-glycylglycine, pH 7·0, 0·8ml.; 50mM-sodium arsenate, 1·0ml.;

Table 1. *Fermentation of glucose during growth of* Zymomonas mobilis

Expt. no.	Glucose utilized (μmoles/ml.)	Ethanol formed (μmoles/ml.)	Molar ratio ethanol/ glucose	Lactate formed (μmoles/ml.)	Molar ratio lactate/ glucose
1	66	106	1·61	1·32	0·02
2	118·5	198·5	1·67	1·35	0·015
3	88	140·8	1·60	—	—

100mM-glutathione, 0·1ml.; 100mM-$NaHSO_3$, 0·2ml.; 40mM-$MgCl_2$, 0·05ml.; 10mM-sodium gluconate, 0·3ml.; 50mM-ATP, 0·2ml.; 2·0mM-NAD, 0·1ml.; 2·0mM-NADP, 0·1ml.; cell extract, 0·1ml.

Pyruvate decarboxylase ('carboxylase') was assayed manometrically in the following system: 0·1M-glycylglycine, pH7·1, 1·5ml.; cell extract, 0·5ml.; 50mM-sodium pyruvate, 1·0ml.; the atmosphere was N_2+CO_2 (95:5) and the temperature was 30°. The formation of acetaldehyde was detected by smell and established as the 2,4-dinitrophenylhydrazone. The CO_2 evolution in this system is not stoicheiometric, an observation which we have also made with the pyruvate decarboxylases of *Zymosarcina ventriculi*, *Zymomonas anaerobia* and *Erwinia amylovora*.

RESULTS

Whole cell experiments

Products of glucose fermentation. Contrary to the reports of previous workers, we were unable to detect the formation of appreciable amounts of lactate by either growing or washed cells during glucose fermentation. The low lactate values have also been confirmed with our strain by Dr J. L. Peel (personal communication). Table 1 records the utilization of glucose and formation of ethanol. The average yield of ethanol (1·64moles/mole of glucose fermented) is lower than that recorded by earlier workers but agrees well with Belaïch & Senez (1965), who used the same strain as us.

Oxidation of glucose by washed cell suspensions. When, early in this work, we observed that *Z. mobilis* could grow in the presence of oxygen, an examination was made of the oxidative capabilities of aerobically and anaerobically grown cells. Both types of cell oxidized glucose without lag and Q_{O_2} values of 60–80 (anaerobic) and 100–140 (aerobic) were recorded. When limited amounts of glucose were used, anaerobically grown cells took up 0·5–0·8μmole of oxygen/μmole of glucose added, and aerobic cells took up 0·7–1·5μmoles of oxygen/μmole of glucose. These differences were consistent and reproducible for cells grown on media containing 2% (w/v) of glucose, and ratios of Q_{O_2} (aerobically grown)/Q_{O_2} (anaerobically grown) were always between 1·3 and 2·5. Recently Belaïch & Senez (1965) reported much smaller differences between aerobically and anaerobically grown cells and we have therefore repeated our experiments with cells grown on their medium. Under these conditions we confirmed their observations, suggesting that the oxidative capabilities of the cells are considerably affected by the medium on which they are grown.

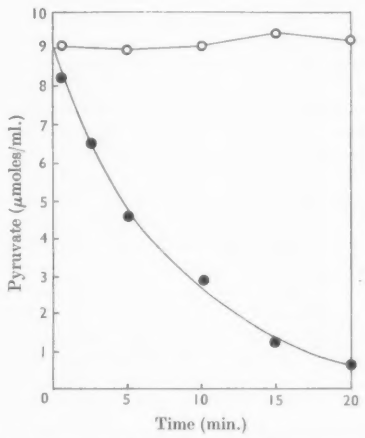

Fig. 1. Pyruvate metabolism under conditions used for the Entner–Doudoroff enzymes assay. Reaction mixtures contained: 0·1M-glycylglycine buffer, pH7·4, 9·6ml.; 0·1M-Na_2AsO_3, 0·4ml.; cell extract, 0·5ml. (8·2mg. of protein); 50mM-sodium pyruvate, 4·0ml.; water to 20ml. ●, No hydrazine; ○, 4·0ml. of 0·56M-hydrazine. Reactions were initiated by addition of pyruvate after incubation at 30° for 5–7min. and samples (1ml.) were withdrawn into 40% trichloroacetic acid (0·3ml.). Pyruvate was estimated in the supernatant after removal of the protein by centrifugation.

Reactions catalysed by cell-free extracts

If an anaerobic Entner–Doudoroff pathway of glucose dissimilation occurs then 6-phosphogluconate should be metabolized to equivalent amounts of pyruvate and glyceraldehyde 3-phosphate in the presence of suitable trapping agents. Appropriate concentrations of hydrazine and arsenite are employed as a routine to prevent further metabolism of glyceraldehyde 3-phosphate and pyruvate

respectively in aerobic systems (Wood & Schwerdt, 1954). We have found that arsenite does not effectively prevent metabolism of pyruvate by extracts of *Z. mobilis* but that hydrazine alone will trap pyruvate (Fig. 1) as well as glyceraldehyde 3-phosphate. The formation of equivalent amounts of triose phosphate and pyruvate from 6-phosphogluconate in the presence of hydrazine is shown in Fig. 2. Approximately 70% conversion of 6-phosphogluconate into pyruvate and triose phosphate occurred; in other experiments between 60 and 70% conversion was consistently observed. Kovachevich & Wood (1955) also observed a similar conversion (64%) of 3-deoxy-2-oxo-6-phosphogluconate into pyruvate and triose phosphate with *Pseudomonas fluorescens*. In the absence of hydrazine pyruvate appears transiently and almost stoicheiometric formation of triose phosphate is observed (Fig. 3); under these conditions the triose phosphate is not metabolized to pyruvate by crude extracts unless supplemented with NAD, ADP and inorganic phosphate. Some inorganic phosphate is usually liberated in the absence of hydrazine. These experiments are consistent with an Entner–Doudoroff pathway. The enzymic sequence common to all pathways of carbohydrate metabolism,

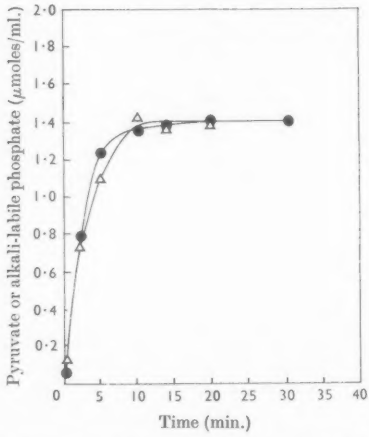

Fig. 2. Formation of pyruvate and glyceraldehyde 3-phosphate from 6-phosphogluconate in the presence of hydrazine. The reaction mixture contained: 0·1 M-glycylglycine buffer, pH 7·0, 9·6 ml.; 0·56 M-hydrazine, 4·0 ml.; water, 0·4 ml.; cell extract, 2·0 ml. (32·1 mg. of protein). It was incubated at 30° for 5 min. before the substrate, 6-phosphogluconate (4·0 ml., 10 mM), was added. Samples (2 ml.) were taken at intervals, added to 40% trichloroacetic acid (0·6 ml.), centrifuged and portions of the clarified solutions used for the analysis of pyruvate (●) and alkali-labile phosphate (△).

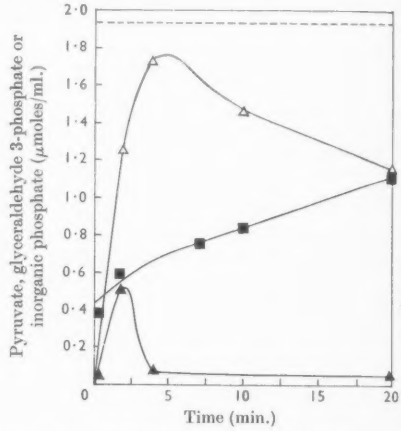

Fig. 3. Formation of pyruvate, glyceraldehyde 3-phosphate and inorganic phosphate from 6-phosphogluconate in the absence of hydrazine. The reaction mixture contained: 0·2 M-glycylglycine buffer, pH 7·3, 2·9 ml.; 10 mM-6-phosphogluconate, 1·2 ml.; water, 1·8 ml.; cell extract, 0·3 ml. (8·0 mg. of protein). ▲, Pyruvate; △, glyceraldehyde 3-phosphate; ■, inorganic phosphate. The horizontal broken line at 1·94 μmoles/ml. indicates the theoretical yield of pyruvate and glyceraldehyde 3-phosphate.

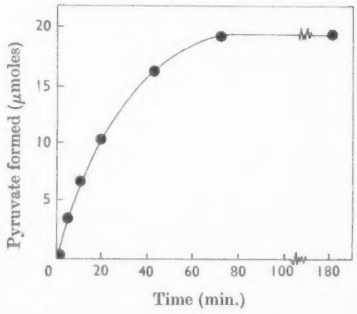

Fig. 4. Formation of pyruvate from glycerate 3-phosphate. The reaction mixture contained: 0·1 M-glycylglycine buffer, pH 7·1, 3·3 ml.; 20 mM-ADP, 1·0 ml.; 40 mM-$MgCl_2$, 0·1 ml.; 0·56 mM-hydrazine, 2·0 ml.; cell extract, 1·6 ml. (12 mg. of protein); it was incubated at 30° for 5 min. for equilibration and then 10 mM-glycerate 3-phosphate (2·0 ml.) was added to initiate the reaction. Samples were treated as described in the legend to Fig. 2.

i.e. the conversion of glycerate 3-phosphate into pyruvate, was also demonstrated (Fig. 4).

$NADH_2$ oxidase. Attempts to determine by spectrophotometric methods the nicotinamide–

adenine dinucleotide specificities of the enzymes involved in the oxidation–reduction balance were initially hindered by the presence in crude cell extracts of a powerful $NADH_2$ oxidase, which could also be determined manometrically. This activity could be removed, however, by centrifuging at 105000*g* for 2hr. Table 2 shows the distribution of $NADH_2$ oxidase between the supernatant and particulate fractions. There was negligible $NADPH_2$-oxidase activity in our extracts.

Nicotinamide–adenine dinucleotide-linked dehydrogenases. The relevant enzymes for maintenance of a balanced oxidation–reduction in the *Z. mobilis* fermentation are glucose 6-phosphate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase and ethanol dehydrogenase.

Glucose 6-phosphate dehydrogenase. This enzyme was non-specific with respect to the nicotinamide–adenine dinucleotide coenzyme; both NAD and NADP are reduced when the supernatants from the ultracentrifuged cell-free extracts are incubated with glucose 6-phosphate. In phosphate buffer reduction of NADP is more rapid than that of NAD; substitution of tris–hydrochloric acid buffer results in the opposite behaviour. The relative activities of the two coenzymes were studied over a range of pH and with a variety of buffers (Table 3).

Ethanol dehydrogenase. Acetaldehyde causes rapid oxidation of $NADH_2$ when incubated in the presence of the supernatant fraction. $NADPH_2$ is not oxidized (Fig. 5*a*). Reversal of the reaction by the addition of ethanol was also demonstrated and a similar coenzyme specificity observed (Fig. 5*b*).

Triose phosphate dehydrogenase. This enzyme was shown to be NAD-specific (Fig. 6). Some impurity (e.g. glucose 6-phosphate) in the commercially available triose phosphates was responsible for the slight reduction of NADP observed. Fructose 1,6-diphosphate aldolase was not detected in these extracts since fructose 1,6-diphosphate did not cause reduction of NAD (by generation of glyceraldehyde 3-phosphate), although addition of crystalline muscle aldolase permitted immediate NAD reduction.

NAD–NADP transhydrogenase. Conclusions about coenzyme specificities could be invalidated by the presence of a transhydrogenase and therefore experiments were carried out to detect this enzyme in extracts (Fig. 7). The results of all such attempts were negative and we conclude that the enzyme is absent from *Z. mobilis*.

Other enzymes. The presence in extracts of hexokinase, gluconokinase, glucose dehydrogenase and pyruvate decarboxylase was established. Neither gluconate dehydrogenase nor fructose 1,6-diphosphate aldolase (Fig. 6) could be detected.

Formation of 3-deoxy-2-oxo-6-phosphogluconate as an intermediate in 6-phosphogluconate catabolism. In the presence of hydrazine, the formation of a

Table 2. *Distribution of $NADH_2$-oxidase activity in extracts of* Zymomonas mobilis

Particulate fraction is the sediment from centrifuging the crude extract at 105000*g* for 2hr. at 0°, taken up in 0·067 M-phosphate buffer, pH 7·0. Each cuvette contained: 0·067 M-phosphate buffer, pH 7·0, 2·5 ml.; enzyme solution (diluted fivefold), 0·1 ml.; 2 mM-$NADH_2$, 0·1 ml.

	Concn. of protein (mg./ml.)	Activity (μmoles of $NADH_2$ oxidized/min./mg. of protein)	
		Specific	Total/ml.
Crude extract	22·20	0·10	2·22
Particulate fraction	12·05	0·17	2·07
Supernatant	7·27	0·03	0·22

Table 3. *Glucose 6-phosphate-dehydrogenase activity of extracts*

Each cuvette contained: buffer, 2·5 ml.; cell extract (105000*g* supernatant), 0·1 ml. (0·71 mg. of protein); 2 mM-NAD or 2 mM-NADP, 0·1 ml.; 10 mM-glucose 6-phosphate, 0·1 ml., the last-named initiating the reactions.

Buffer	Concn. (M)	pH	Activity (ΔE_{340}/min./mg. of protein) NAD	NADP
Pyrophosphate	0·1	8·5	0·13	0·85
Tris–HCl	0·1	8·4	1·09	1·36
		8·0	1·30	1·56
		7·0	1·35	1·05
Glycylglycine	0·1	7·3	1·62	1·62
Phosphate	0·067	7·5	0·29	0·59
Phosphate–citrate	0·05	7·0	0·28	1·24
		6·0	0·18	0·67
Citrate	0·05	6·0	0·02	0·18

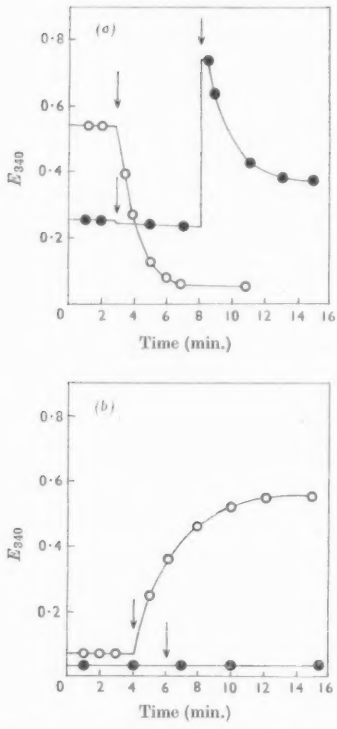

Fig. 5. Coenzyme specificity and reversibility of ethanol dehydrogenase. (*a*) Each cuvette contained: 0·067M-phosphate buffer, pH 7·0, 2·5ml.; cell extract (105000*g* supernatant diluted fivefold), 0·1ml. (0·15mg. of protein); 2mM-$NADH_2$ or 2mM-$NADPH_2$, 0·1ml. ○, $NADH_2$; ●, $NADPH_2$, followed by NAD addition at the indicated time. Reactions were initiated by the addition of acetaldehyde at 3min. Temperature, 23°. (*b*) Each cuvette contained: 0·1M-sodium pyrophosphate buffer, pH 8·5, 0·5ml.; water, 1·45ml.; cell extract (105000*g* supernatant), 0·25ml. (1·9mg. of protein); 3M-ethanol, 0·1ml.; 2mM-NAD or 2mM-NADP, 0·1ml. Ethanol was the last addition made after incubation for 5min. at 22°. ○, NAD; ●, NADP.

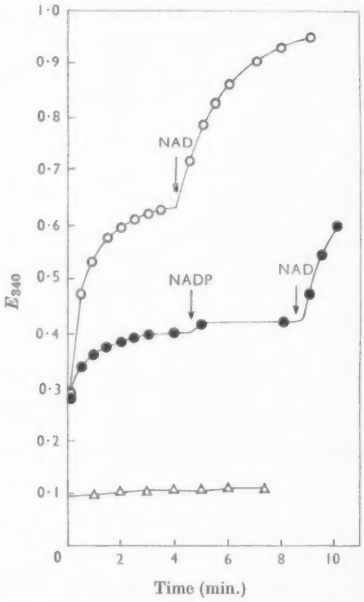

Fig. 6. Coenzyme specificity of glyceraldehyde 3-phosphate dehydrogenase. Each cuvette contained: 0·1M-glycylglycine buffer, pH 7·0, 1·0ml.; 50mM-sodium arsenate, 1·0ml.; 100mM-glutathione, 0·1ml.; 100mM-$NaHSO_3$, 0·2ml.; 40mM-$MgCl_2$, 0·05ml.; triose phosphate esters, 0·2ml. (approx. 16mM), or 10mM-fructose 1,6-diphosphate, 0·2ml.; cell extract (105000*g* supernatant), 0·1ml. (0·71mg. of protein); 2mM-NAD or 2mM-NADP, 0·1ml., as shown. ○, Triose phosphate esters and NAD, followed by a second addition (indicated by arrow) of NAD after 4min.; ●, triose phosphate esters and NADP, followed by a second addition (arrow) of NADP after 4·5min. and NAD after 8·5min. (arrow); △, fructose 1,6-diphosphate and NAD or NADP. Reactions were initiated by addition of the nucleotide. Temperature, 22°.

keto acid from 6-phosphogluconate as determined by the semicarbazide method precedes the formation of pyruvate as determined by the Friedemann & Haugen (1943) technique (curves *A* and *B* respectively, Fig. 8). The difference between total keto acid and pyruvate (curve *C*) represents an intermediary keto acid precursor of pyruvate, presumably 3-deoxy-2-oxo-6-phosphogluconate (MacGee & Doudoroff, 1954). To demonstrate the formation of such a precursor directly, the thiobarbituric acid assay for β-formylpyruvic acid formed by periodate oxidation of 3-deoxy-2-oxo sugars (Weissbach & Hurwitz, 1959; Srinivasan & Sprinson, 1959) was applied.

The formation of 3-deoxy-2-oxo-6-phosphogluconate from 6-phosphogluconate as measured by this method and the subsequent appearance from it of an equivalent amount of triose phosphate (determined as alkali-labile phosphate) in the absence of hydrazine is shown in Fig. 9. Formation of the keto acid was very rapid, approaching the theoretical yield in the initial 2min. of the reaction, and afterwards declining to a value representing about 25% of the theoretical yield. Pyruvate formation under these conditions was transient and very small. Samples of the incubation

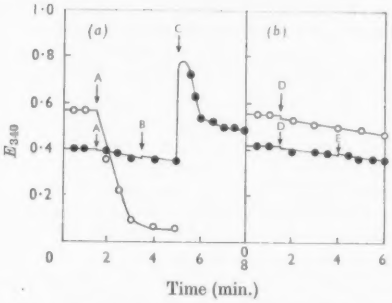

Fig. 7. $NADH_2$ oxidase and transhydrogenase in (*a*) crude cell-free extracts and (*b*) high-speed supernatants (105000*g*). (*a*) Each cuvette contained: 0·067M-phosphate buffer, pH7·0, 2·5ml.; 2mM-$NADH_2$ or -$NADPH_2$, 0·1ml.; crude cell extract (0·1ml., 1·4mg. of protein) was used to initiate the reactions at the arrows indicated by A. After 3·5min. (arrow B) 2mM-NAD (0·1ml.) was added and after 5min. (C) 2mM-$NADH_2$ (0·1ml.) was added to the $NADPH_2$ cuvette. ○, $NADH_2$; ●, $NADPH_2$. Temperature, 24°. (*b*) Each cuvette contained: 0·067M-phosphate buffer, pH7·0, 2·5ml.; 2mM-$NADH_2$ or -$NADPH_2$, 0·1ml.; high-speed supernatant of the cell extract (0·1ml., 0·5mg. of protein) was used to initiate the reactions at the arrows marked D. After 4min. (arrow E) 2mM-NAD (0·1ml.) was added to the $NADPH_2$ cuvette. ○, $NADH_2$; ●, $NADPH_2$. Temperature, 24°.

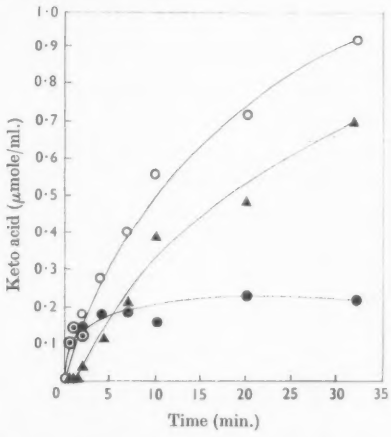

Fig. 8. Formation of keto acids from 6-phosphogluconate in the presence of hydrazine. The incubation mixture contained, in a total of 10ml.: 500μmoles of glycylglycine, pH7·0; 20μmoles of 6-phosphogluconate; 2240μmoles of hydrazine; cell extract (2·6mg. of protein). Temperature, 30°. Samples (1ml.) were removed at intervals into 1ml. of 10% (w/v) perchloric acid and, after centrifuging, the supernatants were analysed as described in the Analytical methods section. No keto acids were detected in the control with 6-phosphogluconate omitted. ○, Total keto acid (*A*); ▲, pyruvate (*B*); ●, difference (*A* – *B*) representing 3-deoxy-2-oxo-6-phosphogluconate (*C*).

mixture taken during the course of the reaction and deproteinized by hot ethanol were chromatographed on paper. Faint spots with the same R_F value as authentic 6-phosphogluconate (0·21–0·33) were found only in samples taken within the first 1·5min. of the reaction. After 1·5min. a spot with the same R_F value as authentic 3-deoxy-2-oxo-6-phosphogluconate (0·42–0·43) was observed but in later samples this disappeared.

DISCUSSION

Isotopic evidence (Gibbs & DeMoss, 1951, 1954; DeMoss, 1953) indicated that the Entner & Doudoroff (1952) route of glucose catabolism operates in *Z. mobilis*, but until 1961 (Ribbons & Dawes, 1961) there was no enzymic evidence to support this belief. *Z. mobilis* is one of the few bacteria that possess a yeast-type pyruvate decarboxylase, and can thus produce ethanol by the reduction of acetaldehyde. The only other ethanol-forming bacteria that possess this enzyme are *Zymomonas anaerobia* (D. J. McGill, D. W. Ribbons & E. A. Dawes, unpublished work), *Zymosarcina ventriculi* (Arbuthnott, Bauchop & Dawes, 1960) and *Erwinia amylovora* (A. Haq & E. A. Dawes, unpublished work) although, unlike the *Zymomonas*, the latter two organisms also metabolize pyruvate by the thioclastic reaction. These various pathways of ethanol formation have been reviewed by Dawes (1963).

The key reactions of the Entner–Doudoroff system are dehydration of 6-phosphogluconate, by the enzyme 6-phosphogluconate dehydratase, to 3-deoxy-2-oxo-6-phosphogluconate, which is split by a specific aldolase to yield 1 mole each of pyruvate and glyceraldehyde 3-phosphate/mole; the triose phosphate is then metabolized to pyruvate by reactions common to glycolysis. Since anaerobic operation of the Entner–Doudoroff system involves a balanced oxidation–reduction between glucose 6-phosphate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase and ethanol dehydrogenase, we were interested in the coenzyme specificities of these enzymes. Of additional interest is the reason why the Embden–Meyerhof pathway of glucose catabolism apparently does not operate, despite the presence of fructose 1,6-diphosphate aldolase (DeMoss & Gibbs, 1952), although we did not detect this aldolase in our extracts. The latter problem was solved when Raps & DeMoss (1962)

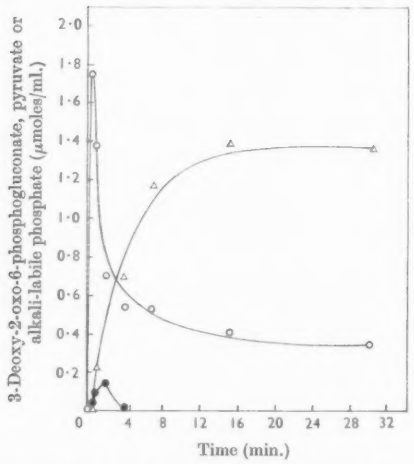

Fig. 9. Formation of 3-deoxy-2-oxo-6-phosphogluconate, pyruvate and alkali-labile phosphate from 6-phosphogluconate in the absence of hydrazine. The incubation mixture contained, in a total volume of 11 ml.: 50 μmoles of glycylglycine, pH 7·0; 20 μmoles of 6-phosphogluconate; cell extract (5·3 mg. of protein). Temperature, 30°. Samples (1 ml.) were removed at intervals into 1 ml. of 10% (w/v) perchloric acid and, after centrifuging, the supernatants were analysed as described in the Analytical methods section (3-deoxy-2-oxo-6-phosphogluconate by the thiobarbituric acid method). No products were detected in the control with 6-phosphogluconate omitted. ○, 3-Deoxy-2-oxo-6-phosphogluconate; ●, pyruvate; △, alkali-labile phosphate.

showed that *Z. mobilis* lacks phosphofructokinase, one of the enzymes unique to glycolysis.

Our experiments show that the enzymes necessary for the production, from 6-phosphogluconate, of 3-deoxy-2-oxo-6-phosphogluconate and its subsequent fission to pyruvate and glyceraldehyde 3-phosphate are present in extracts of the organism, thus substantiating the operation of the Entner–Doudoroff pathway. Further, the 3-deoxy-2-oxo-6-phosphogluconate and glyceraldehyde 3-phosphate finally present at equilibrium account for all of the 6-phosphogluconate metabolized.

Of the three oxidoreductase enzymes involved in the fermentation balance, the reactions catalysed by glucose 6-phosphate dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase presumably reduce the nicotinamide nucleotide and the reduction of acetaldehyde, mediated by ethanol dehydrogenase, re-oxidizes the coenzyme. Since glucose 6-phosphate dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase in other organisms are known to have varying coenzyme specificities, the question of coenzyme specificity in *Z. mobilis* was of some importance. Glucose 6-phosphate-dehydrogenase activity with both NAD and NADP has been observed in *Leuconostoc mesenteroides* (DeMoss, Gunsalus & Bard, 1953), *Pseudomonas fluorescens* (Wood & Schwerdt, 1954) and *Xanthomonas phaseoli* (Hochster & Katznelson, 1958). The last workers were also unable to detect transhydrogenase activity in *X. phaseoli*, although Wood & Schwerdt (1954) found the enzyme in *P. fluorescens* and suggested that the observed activity of glucose 6-phosphate dehydrogenase with NAD in that organism was due to the coupled action of transhydrogenase and an NADP-specific glucose 6-phosphate dehydrogenase. The other oxidative enzyme, glyceraldehyde 3-phosphate dehydrogenase, has been shown to be completely specific for NAD in *P. fluorescens* (Frampton & Wood, 1961) and in *X. phaseoli* (Hochster & Katznelson, 1958).

Our work has confirmed the observation of DeMoss & Gibbs (1952) that glucose 6-phosphate dehydrogenase in *Z. mobilis* is active with both NAD and NADP, and has shown in addition that the glyceraldehyde 3-phosphate and ethanol dehydrogenases are specific for NAD. As in *X. phaseoli* (and unlike *P. fluorescens*), transhydrogenase is absent from *Z. mobilis* and it thus appears likely that, although the glucose 6-phosphate dehydrogenase in the organism is active with both NAD and NADP, only the former is the physiological electron carrier in the balanced oxidation–reduction of anaerobic glucose fermentation. Similar conclusions about the role of NAD in the phosphoketolase-mediated fermentation of glucose by *Leuconostoc mesenteroides* have been reached by DeMoss (1961). Under growth conditions it is possible that the non-specific glucose 6-phosphate dehydrogenase of *Z. mobilis* furnishes $NADPH_2$ for general biosynthesis.

The presence of gluconokinase and the absence of gluconate dehydrogenase suggests that gluconate is metabolized via 6-phosphogluconate and the Entner–Doudoroff enzymes. Although our results do not permit us to conclude whether oxidation of glucose precedes phosphorylation, this seems unlikely since direct oxidation of glucose is not known to be coupled with nicotinamide nucleotide reduction, which is mandatory for ethanol formation.

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Sucrose Utilization by *Zymomonas mobilis*: Formation of a Levan

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1. Molar growth-yield coefficients of *Zymomonas mobilis* for glucose, fructose, glucose plus fructose, and sucrose are reported. Yield coefficients for sucrose are appreciably lower than those for the equivalent concentrations of glucose plus fructose. 2. Only 2·6% of [U-^{14}C]glucose supplied in the growth medium is incorporated into cell substance by *Z. mobilis* utilizing glucose as the energy source. 3. During growth on sucrose a levan is formed. It has been characterized and shown to resemble other bacterial levans. 4. Levan formation from sucrose could be demonstrated with both washed cell suspensions and cell extracts of *Z. mobilis*. 5. Sucrose phosphorylase could not be demonstrated in extracts of the organism.

Tracer studies indicated that *Zymomonas mobilis* ferments glucose via an anaerobic Entner–Doudoroff pathway (Gibbs & DeMoss, 1954) and we have reported that the key enzymes are present in extracts of these cells (Ribbons & Dawes, 1961; Dawes, Ribbons & Large, 1966). As Bauchop & Elsden (1960) pointed out, this is an inefficient pathway when compared with the glycolytic route of ethanol formation in yeast. They demonstrated that the growth yield of *Z. mobilis* per mole of glucose was 8·3, a value which is approximately one-half of that obtained for *Saccharomyces cerevisiae*, but which is accounted for by the provision of only 1 mole of ATP/mole of glucose fermented by an Entner–Doudoroff pathway. Our studies included determination of molar growth yields for *Z. mobilis* when glucose, fructose and sucrose were provided as sources of energy in peptone–yeast extract media, so that we might see how efficiently sucrose is utilized and perhaps distinguish whether hydrolysis or phosphorolysis occurred. Molar growth yields on sucrose were, however, erratic and low and this we have shown to be due to the formation of a levan. The isolation, analysis and synthesis of the levan are described together with the molar-growth-yield experiments that led to its discovery. A preliminary account of this work has been presented to The Biochemical Society (Ribbons, Dawes & Rees, 1962).

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EXPERIMENTAL

Organism, cultural conditions and preparation of cell extracts. Z. mobilis (N.C.I.B. 8938) was maintained, cultured and extracts were prepared from it as described in the preceding paper (Dawes *et al.* 1966) except that, where required, sucrose was added (2%, w/v) to the growth medium as an alternative energy source.

Chemicals and buffer solutions. These were as described in the preceding paper (Dawes *et al.* 1966). [U-^{14}C]Glucose was obtained from The Radiochemical Centre, Amersham, Bucks.

Analytical methods

Chemical estimations. Fructose was determined with the resorcinol reagent (Umbreit, Burris & Stauffer, 1957). Total carbohydrate was estimated by the anthrone method of Trevelyan & Harrison (1952). For other estimations see Dawes *et al.* (1966). Elemental analyses were performed by Mr J. M. L. Cameron, Chemistry Department, University of Glasgow.

Levan determination. (*a*) Levan was estimated turbidimetrically in 1cm. cells with a Spekker absorptiometer by using filters H503 and 603; linearity of levan concentration against drum reading was observed up to 2·5mg. (15·43μ-moles of fructose equivalent) of levan/ml., when the value was 0·615. Thereafter increments of drum readings decreased as the levan concentration was further raised, e.g. 4·25mg. of levan/ml. gives a reading of 0·91. (*b*) Levan was estimated by measurement of the amount of total fructose that is precipitated by 75% ethanol, after washing the precipitate in 75% ethanol. Results have been expressed as μmoles of fructose equivalent, assuming a mean residue weight of 162.

Cell fractionation. The procedures of Roberts, Abelson, Cowie, Bolton & Britten (1957) were used.

Isotopic procedures. The radioactivity of the fermentation CO_2 trapped in alkali was measured by precipitation of replicate portions as $BaCO_3$ by the methods described by Sakami (1955). The radioactivity of cells was determined by spreading portions (0·1 and 0·2 ml.) of suitably diluted suspensions on stainless-steel planchets having an effective raised area of 1·5 cm.^{2} (Dawes & Holms, 1958). Cell fractions were plated in a similar manner. Ethanol was steam-distilled from supernatants at neutral pH and collected in dichromate (Neish, 1952). The acetate formed was distilled, titrated with 0·05 N-NaOH and portions (0·1 and 0·2 ml.) of the resulting sodium acetate solution were spread on planchets.

All estimations were made at or corrected to infinite thinness by using an end-window Geiger–Müller tube and an Ekco automatic scaler type N530 G. Counts were collected for a period sufficient to ensure that the counting error was less than ±2%. Corrections were applied for background and coincidence.

Paper chromatography. Descending paper chromatograms were developed in the solvent systems: (*a*) water-satd. phenol+1% of aq. NH_3 (sp.gr. 0·88); (*b*) butan-1-ol–acetic acid–water (4:1:5, by vol.); (*c*) propan-1-ol–ethyl acetate–water (7:1:2, by vol.) (Feingold, Avigad & Hestrin, 1956); (*d*) butan-1-ol–ethanol–water (5:1:4, by vol.; upper phase). Carbohydrates were detected with ammoniacal $AgNO_3$ (Partridge, 1948; Trevelyan, Procter & Harrison, 1950), aniline oxalate (Horrocks & Manning, 1949), urea–phosphate (Wise, Dimler, Davis & Rist, 1955) and triphenyltetrazolium chloride (Wallenfels, 1950).

Gas-phase chromatography. This was carried out with a Pye Argon Gas Chromatograph with a ^{90}Sr detector.

Molar growth yields. The yields of *Z. mobilis* on glucose, fructose and sucrose were generally determined turbidimetrically in a manner identical with that employed by Bauchop & Elsden (1960). Occasionally direct weight measurements were made on centrifuged and washed cultures as described by the same authors.

Spectrophotometry. Ultraviolet and visible spectra were established as before (Dawes *et al.* 1966). Infrared spectra were determined with a Perkin–Elmer Infracord with liquid paraffin (Nujol) mulls.

Ultracentrifuge. A Spinco model E ultracentrifuge was used. The polysaccharide concentration was 2·7 mg./ml. (water, solvent) and the rotor speed 11573 rev./min. A schlieren angle of 55° and exposures of 15 sec. at 4 min. intervals were employed. The temperature was 17·4°.

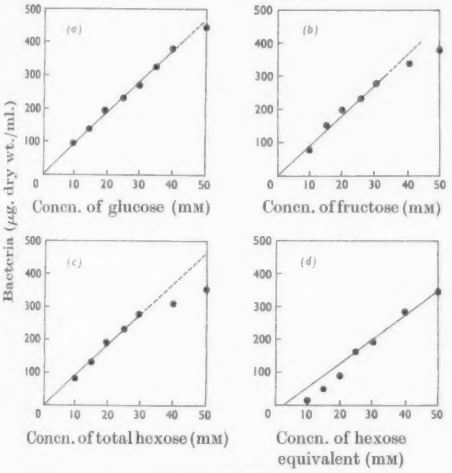

Fig. 1. Relationship between concentration of energy source and cell yield of *Z. mobilis*. Growth was measured turbidimetrically and the experimental protocol was as described by Bauchop & Elsden (1960). (*a*) Glucose; (*b*) fructose; (*c*) equimolar mixture of glucose and fructose; (*d*) sucrose. The points are the average values of six individual determinations for each concentration.

RESULTS

Molar growth-yield coefficients

The relationship between total bacterial growth and the concentration of energy source provided is shown in Fig. 1. Glucose and fructose and an equimolar mixture of glucose and fructose allow growth yields (g. dry wt. of bacterial cells/mole of hexose equivalent) of 9·32, 9·21 and 9·16, whereas

Table 1. *Growth-yield coefficients of* Zymomonas mobilis *with glucose and sucrose as sources of energy*

Cultures were grown in 1% Bacto-Peptone–1% yeast-extract media supplemented with the energy source in 250 ml. conical flasks. The culture volume was 155 ml., and was inoculated with a loop of an exponential-phase culture developing on media of similar energy source which was limiting total growth. Cell densities were determined turbidimetrically and by direct weighing.

Energy source	Concn. (mM)	Total cell yield (mg.)		Yield coefficient	
		Turbidimetric	Direct weighing	Turbidimetric	Direct weighing
Glucose	29·0	37·2	39·2	8·30	8·71
Sucrose	14·5	28·1	31·0	6·22	6·90
Sucrose	14·5	28·5	34·5	6·32	7·45

sucrose gives a yield of only 7·36, which varies from experiment to experiment between 5·35 and 8·0. A direct determination of the weight of bacterial cells is illustrated in Table 1, where a consistently lower yield of cells is obtained on sucrose than on glucose. The yield of ethanol from these substrates was 1·68 (glucose) and 1·35 (sucrose) moles/mole of hexose equivalent.

We had previously demonstrated that the energy source (with glucose) is not incorporated appreciably into cellular materials (Table 2); hence it was considered likely that other metabolic products were formed from sucrose as no sucrose could be detected after fermentation. The values which trace the fate of the carbon from [U-^{14}C]glucose after fermentation agree favourably with those obtained recently by Belaïch & Senez (1965) and demonstrate that, even under conditions of excess of energy source, there is little incorporation into cellular material. Assuming a carbon content of cells of 50%, then the ratio of the specific activity of cellular carbon to the substrate glucose carbon is 0·83:1·0, i.e. considerable dilution of the glucose into cellular material has not occurred. The distribution of the ^{14}C in the cells was also fairly uniform, indicating that it is not accounted for by a specific storage material or by structural carbohydrate as in *Zymosarcina ventriculi* (E. A. Dawes & D. W. Ribbons, unpublished work) (Table 3). The carbohydrate content of the organism is, in fact, very low, representing only some 3% of the dry weight.

Isolation and analysis of the levan

Isolation. Centrifugation of sucrose cultures of *Z. mobilis* for 1–2min. at 15000*g* yielded a cell pellet and an opalescent supernatant fluid which could be clarified by centrifugation at 25000–30000*g* for 30min. The pellets obtained were translucent gels, and were easily resuspended and precipitated by further centrifugation. Isolation of sufficient material for its chemical characterization was achieved as follows.

Four cultures (8l. each) of *Z. mobilis* on 2%

Table 2. *Fate of* [*U*-^{14}C]*glucose during growth of* Zymomonas mobilis *in glucose-limiting cultures*

A culture medium (53ml., 2% glucose, 1% Bacto-Peptone and 1% yeast extract) containing [U-^{14}C]glucose (3×10^6 counts/min.) was inoculated with a stationary-phase culture of *Z. mobilis* (5ml.). A sample (5ml.) was immediately withdrawn for chemical and radiochemical analysis and the rest of the culture harvested after 16·5hr., washed twice with the same volume of water and then with 25ml. of water. The cells were suspended in water and made up to 50ml., giving a cell density 581μg. dry wt./ml. The culture supernatant (1ml.) was steam-distilled and excess of potassium dichromate oxidizing mixture (Neish, 1952) added to the distillate (4ml.). After standing for 1hr. at 22° the acetic acid formed was collected by steam-distillation in a volume of 25ml. and portions were titrated with CO_2-free 0·05N-NaOH and plated and counted as sodium acetate. The radiochemical yield of CO_2 was calculated from the yield of ethanol in the fermentation, i.e. 1·6moles/mole of hexose.

Fermentation yields			Radiochemical distribution (counts/min.)			
Glucose fermented (m-moles)	Ethanol formed (m-moles)	Cell yield (mg.)	Glucose utilized	Ethanol	CO_2	Cells
4·67	7·45	20·91	$2·6\times10^6$ (100%)	$1·32\times10^6$ (50·8%)	$7·6\times10^5$ (26·7%)	$6·7\times10^4$ (2·6%)

Table 3. *Distribution of* ^{14}C *in cellular fractions after growth on* [*U*-^{14}C]*glucose*

Cells from the experiment of Table 2 were used. Duplicate portions (5ml., 5·8mg. dry wt.) of a cell suspension in water were centrifuged and subjected to the fractionation procedure of Roberts *et al.* (1957).

Fraction	Radioactivity (counts/min.)		Percentage of total radioactivity	
Whole cells	27000		100	
Cold trichloroacetic acid (5%)	1920	1699	7·1	6·25
Ethanol (75%, v/v)	4280	5210	15·8	19·2
Hot trichloroacetic acid (5%)	5600	6240	20·8	23·0
Residue	9788	10080	36·0	37·2
Recovery			79·7	85·65

sucrose–Bacto-Peptone–yeast-extract medium were grown into the stationary phase. Centrifugation at 50000*g* with a fast flow rate (500ml./min.) on the Sharples Super-Centrifuge yielded a cloudy supernatant which was almost devoid of bacterial cells. The supernatants were cooled to 0–2° and centrifuged in batches at 27000*g* for 30min. on an MSE angle 13 centrifuge. Supernatants were discarded and the sedimented gel was taken up into water (1l.) and resedimented at 27000*g* for 90min.; the process was repeated. Suspension of the pellets in 600ml. of water yielded a grey–brown opalescent solution which was treated with 1800ml. of industrial spirit. A brown gum immediately separated to the bottom of the beaker, the cloudy white supernatant was clarified at 20000*g* for 5min. and the pellets were combined with the gum. Solution of the solid in 300ml. of water and addition of ethanol to 75% (v/v) reprecipitated the levan, which was taken up in 250–300ml. of water, filtered and dialysed for 24hr. against distilled water at room temperature. Filtration of the dialysed preparation and freeze-drying produced a fluffy white material which was dried over phosphorus pentoxide. The yield was 4·5g.

Characterization of the polysaccharide as a fructosan. Fructose was the only sugar that could be detected in this hydrolysate by paper chromatography with solvents (*a*) and (*b*). Polysaccharide (300mg.) was heated in 0·02N-sulphuric acid (20ml.) at 100° for 45min. The solution was neutralized with barium carbonate and concentrated to dryness under diminished pressure. The resulting syrup was thoroughly dried in a vacuum desiccator over phosphorus pentoxide and treated with acetone in the presence of conc. sulphuric acid as described by Bell (1947) to give 2,3:4,5-di-isopropylidene-D-fructose: 250mg.; m.p. and mixed m.p. with authentic material, 93·5–95°, $[\alpha]_D - 34·5°$ (*c* 0·8 in water). Analysis of a sample of levan which had been pre-dried at 60° *in vacuo* for 24hr. by the method of Roe (Umbreit *et al.* 1957) showed 94·5% of anhydrofructose residues. Nitrogen analysis gave <0·01%.

Periodate oxidation. Fructosan (15·3mg.) was dissolved in sodium metaperiodate (0·07M; 3ml.) and the solution left at room temperature, the periodate uptake being determined from time to time by Aspinall & Ferrier's (1957) method. There was an initial rapid consumption of 0·96mole of oxidant (within 5hr.), rising finally to a value of 1·00mole/mole of anhydrofructose (24hr.). These results are consistent with a formula for the fructosan consisting entirely of fructofuranose residues linked 2,1 or 2,6 or both.

Partial acidic hydrolysis. Fructosan (20mg.) was partially hydrolysed in 0·01N-sulphuric acid (2ml.) at 60° for 1hr. After neutralization on IR-4B resin and concentration the hydrolysate was examined by paper chromatography with solvent (*c*). For comparison, samples of dahlia inulin and levan of perennial rye-grass tops were treated in exactly the same way. *Zymomonas* fructosan gave a series of oligosaccharides (detected up to the pentasaccharide) which resembled in R_F value and in behaviour towards triphenyltetrazolium chloride spray (cf. Feingold *et al.* 1956) the series from perennial rye-grass levan. In contrast the oligosaccharides from dahlia inulin had different R_F values and could not be detected with triphenyltetrazolium chloride, though they were readily detected with alkaline silver nitrate (Trevelyan *et al.* 1950).

Methylation of levan. Levan (1·0g.) was stirred with water (25ml.) until it dissolved and the flask was then cooled in an ice bath. Sodium hydroxide (30%, w/v; 45ml.) and dimethyl sulphate (15ml.) were added simultaneously in portions with vigorous stirring over 5hr. The reaction mixture was allowed to reach room temperature and stirred overnight. The same reagents were added similarly the following day (at room temperature) and the procedure was repeated a further three times. Acetone was added from time to time to maintain complete solution. Finally the mixture was stirred at 60° for 1hr., cooled, neutralized with 10N-sulphuric acid, and the methylated polysaccharide extracted with chloroform. The combined extracts were washed once with water, evaporated under diminished pressure to small volume and poured into excess of light petroleum (b.p. 60–80°). The precipitate was removed on the centrifuge, washed thoroughly with light petroleum, and dried *in vacuo* at 60° to give a buff amorphous solid (0·9g.), which showed no hydroxyl group absorption in the infrared spectrum [Found: OCH_3, 45·6; fully methylated fructosan requires OCH_3, 45·5%. $[\alpha]_D - 59·4 \pm 1°$ (*c* 0·6 in chloroform)].

Paper chromatography of hydrolysed methylated levan. The material was hydrolysed by the method of Arni & Percival (1951) and the resulting syrup compared with authentic specimens of methylated fructoses by chromatography in solvent (*d*) with urea–phosphate spray. The hydrolysate contained three components. The first (R_F relative to tetramethylfructose, 0·65) moved at the same rate as authentic 3,4-di-*O*-methylfructose. The second component (relative R_F 0·84) moved at the same rate as authentic 1,3,4-tri-*O*-methylfructose. Authentic 3,4,6-tri-*O*-methylfructose on the same chromatogram could be distinguished in that (i) it travelled a little faster, and (ii) it gave a very strong reaction with triphenyltetrazolium spray, whereas the tri-*O*-methylfructose in the hydrolysate, like the authentic 1,3,4-tri-*O*-methylfructose, gave no reaction. The third component moved at

the same rate as authentic 1,3,4,6-tetra-*O*-methylfructose and gave a similar inky blue–green colour with urea–phosphate spray. Similar amounts of di- and tetra-*O*-methylfructoses were present, as judged from spot intensities, with larger amounts (six to ten times as much) of the tri-*O*-methylfructose.

Gas-phase chromatography of methanolysed methylated levan. The material (10mg.) was treated in a sealed Pyrex tube with methanolic hydrogen chloride (2·5%; 1·5ml.) at 60° for 3hr. After neutralization with silver carbonate the filtrate was concentrated to a syrup and compared by gas-phase chromatography with authentic di-, tri- and tetra-*O*-methylfructoses which had been treated in the same way. The systems used were (i) 15% butanediol succinate polyester on Celite at 175° and (ii) 10% polyphenol on Celite at 200° (Aspinall, 1963). The two tri-*O*-methylfructoses could be readily distinguished (Aspinall, 1963) and peaks corresponding to those given by the authentic 1,3,4-isomer were found to be present. No 3,4,6-tri-*O*-methylfructose was detected. Other peaks on the chart indicated the presence of 3,4-di- and 1,3,4,6-tetra-*O*-methylfructose derivatives.

Ultracentrifugal analysis. Concentrated solutions of the levan showed one major and one slower minor peak. Results obtained gave $S_{20,w}$ as 390×10^{-13} sec.

Formation of levan

Formation during growth. Yields of levan from 2% sucrose cultures varied between 100 and 200mg./l. Neither glucose nor fructose supported levan synthesis. Raffinose was not tested as a growth substrate.

Formation by washed cell suspension. Fig. 2 demonstrates the course of sucrose degradation and levan formation by non-growing cells. This experiment clearly shows that glucose is utilized faster than the fructose portion, and that levan synthesis stops when, at the most, the sucrose concentration is 500μg. (1·46μmoles)/ml. (i.e. the difference between total carbohydrate and fructose present). After about 4hr. incubation only free fructose is left and this continues to be metabolized by the cells without levan formation.

Enzymic synthesis. Levan is readily synthesized by crude extracts of *Z. mobilis*, without added primer (Fig. 3). Approximately 10% of the sucrose

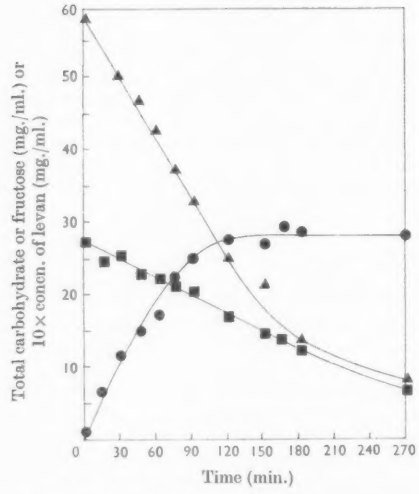

Fig. 2. Synthesis of levan by washed cell suspensions. A washed cell suspension (15ml., 11·7mg./ml.) was obtained from a 350ml. sucrose culture and added to phosphate–citrate buffer, pH5·6 (15ml.), and 15% (w/v) sucrose solution (15ml.), all previously incubated at 30°, and samples (2ml.) were taken as indicated. Samples were immediately centrifuged for 1–2min. on a bench centrifuge (2000*g*) to remove cells and subjected to analysis of levan (turbidimetrically) (●), total carbohydrate (including levan) (▲) and total fructose (■). Note that the levan scale differs by a factor of 10.

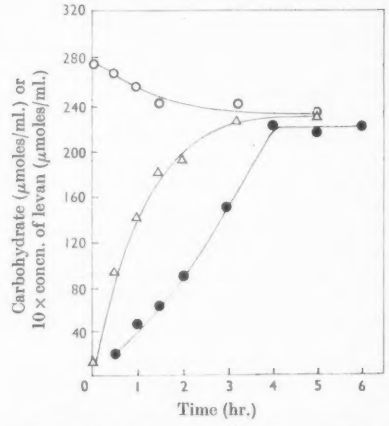

Fig. 3. Enzymic formation of levan. The reaction mixture contained: citrate–phosphate buffer, pH5·0 (3ml.); 15% (w/v) sucrose (3ml.); enzyme solution (crude extract, 3ml., containing 74mg. of protein). Temperature, 32°. Samples (1ml.) were taken for analysis as indicated. ●, Levan formation (turbidimetric); ○, total non-levan carbohydrate (anthrone), i.e. carbohydrate soluble in 75% ethanol; △, reducing sugar formed. Controls containing 75% of ethanol were included in the anthrone assays (Binnie, Dawes & Holms, 1960). Reducing sugar was determined before ethanol treatment on the supernatant after deproteinization by the method of Somogyi (1930).

Table 4. *Levan formation by extracts of* Zymomonas mobilis

Each test tube contained: citrate–phosphate buffer of the indicated pH (3 ml.); sucrose or raffinose, 15%, as indicated (3 ml.); cell-free extract (3 ml.; 21 mg. of protein/ml.). Controls without added sugars were run at the three pH values so that increases in turbidity due to protein denaturation could be followed, and subtracted from the reaction tubes. Turbidity due to protein denaturation amounted to approx. 25% of the values obtained in reaction tubes. Figures in the columns represent (*a*) levan formed (measured turbidimetrically), (*b*) reducing sugar formed and (*c*) total fructose soluble in 75% ethanol.

	Sucrose, pH 4·6			Sucrose, pH 5·0			Sucrose, pH 6·0			Raffinose, pH 5·0		
Time (min.)	Levan formed (μmoles/ml.)	Reducing sugar (μmoles/ml.)	Total fructose (μmoles/ml.)	Levan formed (μmoles/ml.)	Reducing sugar (μmoles/ml.)	Total fructose (μmoles/ml.)	Levan formed (μmoles/ml.)	Reducing sugar (μmoles/ml.)	Total fructose (μmoles/ml.)	Levan formed (μmoles/ml.)	Reducing sugar (μmoles/ml.)	Total fructose (μmoles/ml.)
2		16·2	179·0		14·1	160·0		4·5	164·5		7·1	73·7
25	16·2			11·5			1·2			4·3		
30		83·7	154·0		91·2	148·0		62·5	163·0		31·0	67·5
50	23·1			19·3			6·2			10·8		
60		131·0	136·0		142·0	157·0		115·5	168·0		55·3	62·5
80	42·9			34·3			12·0			29·0		
90		175·0	136·0		181·2			155·0	156·0		71·2	65·0
110	66·4			43·5			13·9			30·9		
120		180·0	126·2		185·0	145·0		169·0	152·5		82·5	60·0
190	117·3			83·3			26·2			46·3		
300	123·5	218·5	122·0	115·4	225·0	141·0	48·8	204·0		40·7	106·5	61·7
360	118·5	205·0	118·0	118·5	226·0	136·0	52·5	231·0	156·0	40·1	125·0	

utilized by extracts is converted into levan, the remainder of the sucrose being hydrolysed to glucose and fructose. As might be expected, levan formation does not occur at a linear rate, as judged by the methods we have employed to assay its formation, namely increased turbidity during incubation, analysis of total carbohydrate that is insoluble in 75% ethanol (not shown in this experiment), and also by difference from the analysis of total carbohydrate that is soluble in 75% ethanol (Fig. 3). This latter value also shows a lag before it starts to decline. The molecular size which must be attained before these turbidimetric or precipitation methods of analysis are accurate is not known. The reaction mixtures were not investigated further for other products such as fructosylsucrose. However, there seems little reason to doubt that this levan sucrase differs from those previously studied in *Acetobacter levanicum* (Hestrin, Feingold & Avigad, 1956) and *Bacillus subtilis* (Dedonder, 1960). The pH optimum is about 5 and the specificity of reaction extends to other fructofuranosyl-α-D-aldosides, e.g. raffinose (Table 4).

In crude unsupplemented extracts the main products of sucrose metabolism are glucose and fructose. Addition of inorganic phosphate (or its use in buffer systems) neither stimulates nor inhibits the rate of sucrose metabolism, nor do the products alter appreciably. It seems therefore that phosphorolysis plays little part in sucrose degradation by *Z. mobilis*. Only when ATP is added to extracts does further metabolism of the hexoses occur. Table 5 records the formation of pyruvate from glucose, fructose and sucrose by extracts supplemented with ATP and NAD. The rate of sucrose degradation is relatively low, irrespective of the energy source used in the growth media, a fact noted earlier when washed suspensions of *Z. mobilis* were allowed to ferment glucose, fructose or sucrose (Table 6). However, this is a property which may easily vary with pH value. The rates of oxygen consumption by whole cells metabolizing

Table 5. *Formation of pyruvate from sucrose, glucose and fructose*

Reaction mixtures contained: 0·2M-glycylglycine, pH 7·3 (2·0ml.); 20mM-carbohydrate as indicated (1·0ml.); 20mM-NAD (0·1ml.); 40mM-$MgCl_2$ (0·3ml.); 50mM-ATP (1·0ml.); 0·56M-hydrazine (1·2ml.); cell-free extract (0·5ml., 12·5mg. of protein), the last-named addition initiating the reactions. Temperature, 30°. Samples (1ml.) were taken at intervals for analysis of pyruvate and carbohydrate (anthrone method).

	Carbohydrate substrate					
	Glucose		Fructose		Sucrose	
Time (min.)	Pyruvate (μmoles/ml.)	Carbohydrate (μmoles/ml.)	Pyruvate (μmoles/ml.)	Carbohydrate (μmoles/ml.)	Pyruvate (μmoles/ml.)	Carbohydrate (μmoles/ml.)
1·0	0·02	3·73	0·10	3·32	0·08	9·40
2·5	0·08	3·21	0·20	3·28		9·00
5	0·39	2·52	0·33	2·60		9·00
10	0·52	1·74	0·52	1·64	0·19	8·95
20	0·76	0·53	0·79	0·39	0·25	
30	1·26	0·58	1·16	0·40	0·36	8·60

Table 6. *Relative rates of carbohydrate utilization by washed suspensions of* Zymomonas mobilis *grown with different carbohydrates*

Carbon dioxide formation was measured manometrically under an N_2+CO_2 (95:5) atmosphere. Each flask contained: 0·1M-citrate buffer, pH 6·6 (1·0ml.); substrate (1·0ml., 10μmoles); cell suspension (1·0ml.). Temperature, 30°. Dry wt. (mg.) of cells per flask: (*a*) 3·3; (*b*) 5·5; (*c*) 7·2; (*d*) 4·2; (*e*) 4·2; (*f*) 8·6. Rates are expressed as μl. of CO_2 evolved/hr./mg. bacterial dry wt. (Q_{CO_2}).

	Fermentation rates (Q_{CO_2})					
Carbohydrate in growth medium ...	Glucose		Fructose		Sucrose	
Fermentation substrate	(*a*)	(*b*)	(*c*)	(*d*)	(*e*)	(*f*)
Glucose	319	317	346	358	293	206
Fructose	54·5	60·0	154	178	137	77
Sucrose	3·6	3·6	14·7	6·6	12·3	24·4

sucrose were also much slower than for glucose or fructose.

DISCUSSION

The observed low values for the molar growth yield of *Z. mobilis* utilizing sucrose, which led to the discovery of the levan, can be explained by the diversion of some of the energy source to fructan formation. Variations in the quantity of levan formed would account for the differences in yield coefficient recorded. Furthermore, the determination of yield coefficient was subject to variation due to (*a*) the light-scattering properties of the levan and (*b*) the ease with which it is centrifuged when bacterial dry weights are measured.

Utilization of sucrose could conceivably occur by hydrolysis or by phosphorolysis. A sucrose phosphorylase which catalyses reaction (1) is present in

$$\text{Sucrose} + P_i \rightleftharpoons \text{glucose 1-phosphate} + \text{fructose} \quad (1)$$

Pseudomonas saccharophila and *Leuconostoc mesenteroides* (Doudoroff, 1955), but no evidence for the activity of this enzyme in *Z. mobilis* could be obtained. An organism which possesses sucrose phosphorylase is energetically more efficient in its utilization of sucrose than one which does not, for phosphate is introduced into the glucose molecule without the expenditure of ATP. Consequently, where the Entner–Doudoroff pathway operates, the ATP yield by phosphorolysis would be 3, as opposed to 2moles/mole of sucrose by hydrolysis. The molar growth yield on sucrose should thus be greater than that on the equivalent concentration of glucose plus fructose by a factor of 1·5 if phosphorolysis occurs. The observed yields were, in fact, substantially lower and, together with the enzymic evidence, it is concluded that *Z. mobilis* utilizes sucrose by a hydrolytic mechanism.

Our results show clearly that levan formation is accompanied by hydrolysis to glucose and fructose, but we have not established whether an invertase is present. Levan sucrase catalyses the reaction (2),

$$\underset{\text{Sucrose}}{n\,C_{12}H_{22}O_{11}} + \underset{\text{acceptor}}{ROH} \longrightarrow \underset{\text{levan}}{H(C_6H_{10}O_5)_nOH} + \underset{\text{glucose}}{n\,C_6H_{12}O_6} \quad (2)$$

where the acceptor is fructose and the donor either sucrose or raffinose; if water is the acceptor, then the products will be fructose and glucose. With crude extracts the products of sucrose metabolism are glucose, fructose and levan; the hexoses are not further metabolized unless ATP is added. The levan sucrase alone could thus account for our findings.

Bacterial levans are a family of high-molecular-weight fructans in which D-fructofuranosyl units are joined mainly by 2,6-linkages but with about every ninth unit carrying a 2,1-branch-point (Hestrin, 1959), the glycosidic configuration probably being β throughout. *Z. mobilis* levan would appear to be a typical member of this family in every respect. Periodate oxidation revealed that if the sugar units were in the furanose ring form, then the glycosidic linkages were attached exclusively to primary hydroxyl groups. Partial hydrolysis showed that the major linkage was 2,6, that large numbers of such linkages were consecutive and that many (probably all) of the units were indeed in the furanose ring form. Methylation analysis gave results that agreed with these conclusions and showed further that 2,1-linkages were present in about the usual proportions for bacterial levans, and that all these linkages were present at branch-points.

The specific optical rotation of the methylation polysaccharide confirmed the general similarity to other bacterial levans. The results of the partial hydrolysis study, together with the value for the optical rotation of the methylated derivative, suggest that most, if not all, of the glycosidic linkages have the β-configuration. The sedimentation coefficient (390s) was similar to the values quoted (Feingold & Gehatia, 1957) for levans with molecular weights of the order of 10^7.

Dr G. O. Aspinall very generously provided reference samples of fructans and methylated sugars, and carried out the analyses by gas chromatography. We thank Professor Sir Edmund Hirst, C.B.E., F.R.S., for his interest. Dr J. Pitts kindly provided the ultracentrifuge data. We are grateful to Mrs I. Thomson and Mr J. Hastings for technical assistance.

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The Purification from Rat Liver of a Nuclease Hydrolysing Ribonucleic Acid and Deoxyribonucleic Acid

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1. The purification of a nuclease from rat-liver mitochondria is described. The mitochondria are rendered soluble by treatment with Triton X-100 and, after fractionation with ammonium sulphate and acetone, the active fraction is further purified by chromatography on DEAE-cellulose and Sephadex G-75 to give a purification of over 700-fold. 2. The purified enzyme was only very slightly contaminated with deoxyribonuclease II, phosphodiesterase and phosphomonoesterase. The individual activities of these enzymes did not exceed 0·1% of the activity of the liver nuclease. 3. The purified enzyme attacked RNA more rapidly than denatured DNA and hydrolysed native DNA more slowly than denatured DNA. 4. There is some evidence to suggest that the nucleolytic activity of the purified preparation towards native DNA, denatured DNA and RNA is associated with a single protein. 5. The enzyme is relatively labile but is stabilized in the presence of 20% (w/v) glycerol or 10mM-2-mercaptoethanol.

The presence of alkaline deoxyribonuclease in mammalian tissues has been demonstrated by Shack (1957), and Beaufay, Bendall, Baudhuin & de Duve (1959) showed that in rat liver this activity is largely localized in the mitochondria. The alkaline deoxyribonuclease of rat liver has been shown to hydrolyse denatured DNA preferentially (Burdon, Smellie & Davidson, 1964), suggesting that the enzyme may be similar to the lamb-brain endonuclease described by Healy, Stollar, Simon & Levine (1963) which has an absolute specificity towards denatured DNA. In the present studies the deoxyribonuclease from rat liver has been purified more than 700-fold without showing any increase in specificity towards denatured DNA.

EXPERIMENTAL

Materials. DNA from Landschutz ascites-tumour cells and calf thymus was used. DNA was prepared from calf thymus glands by the procedure of Kay, Simmons & Dounce (1952). This method was also employed for the isolation of DNA from the sediment obtained on centrifuging disrupted Landschutz ascites-tumour cells at 105000*g* for 1hr. (Keir, Binnie & Smellie, 1962). The purified DNA was dissolved in water at a final concentration of 2mg./ml. for subsequent use. Thermally denatured DNA was prepared by heating a solution of DNA (2mg./ml. in water) in a boiling-water bath for 10min. followed by rapid cooling in an ice bath.

Highly polymerized yeast RNA was purchased from the British Drug Houses Ltd., Poole, Dorset, and dissolved in water to give a solution containing 2mg./ml. Yeast s-RNA,* prepared by the method of Bell, Tomlinson & Tener (1964), was kindly supplied by Dr D. Bell of this Department. The s-RNA, as judged by its behaviour in the ultracentrifuge and by the hyperchromicity observed on heating, was probably double-stranded. *p*-Nitrophenyl phosphate (disodium salt) was purchased from the British Drug Houses Ltd. and thymidine 5′-*p*-nitrophenyl phosphate (monosodium salt) from the California Corp. for Biochemical Research, Lucerne, Switzerland. Thymidine 3′-*p*-nitrophenyl phosphate was synthesized by the method of Turner & Khorana (1959). Triton X-100 was purchased from Rohm and Hass, Philadelphia, Pa., U.S.A., 2-mercaptoethanol from L. Light and Co. Ltd., Colnbrook, Bucks., and bovine serum albumin from the Armour Pharmaceutical Co., Eastbourne, Sussex. DEAE-cellulose (DE11 fibrous powder with a nominal capacity of 1m-equiv./g.) was purchased from Reeve Angel and Co. Ltd., London, E.C. 4, and was prepared according to the method of Peterson & Sober (1962). The DEAE-cellulose was equilibrated with buffer of 5 times the concentration of the eluting buffer and packed under pressure (approx. 15cm. Hg). The column was then washed with 10 times its bed volume of the eluting buffer. The pH of the effluent was checked to ensure that it was the same as that of the eluting buffer.

Sephadex G-75 (bead form) was purchased from Pharmacia, Uppsala, Sweden. The gel was pretreated and packed as described by Flodin (1962). The packed column (1·7cm. × 106cm.) was washed with 10mM-imidazole–HCl buffer, pH 6·8, made 5mM with respect to 2-mercaptoethanol, until a stable column had formed. Before use the void volume of the column was determined by measuring the volume of eluent required to move peak concentrations of Blue Dextran 2000 (mol.wt. 2×10^6; obtained from Pharmacia) from the top to the bottom of the column. Other reagents were A.R. grade except for tris and imidazole, which were laboratory reagents.

* Abbreviation: s-RNA, transfer or 'soluble' RNA.

Enzyme assays. One unit of activity is defined as the amount of enzyme which releases 1 μmole of nucleotide or 1 μg.atom of nucleotide phosphorus/hr. The specific activity is expressed as units/mg. of protein.

(*a*) Assay of liver nuclease. Activity was measured by following the ultraviolet absorption of the acid-soluble products as in the procedure of Lehman, Bessman, Simms & Kornberg (1958). The assay medium, containing 300 μg. of denatured DNA, 25 μmoles of imidazole–HCl buffer, pH 6·8, 0·25 μmole of $MgCl_2$ and 5 μmoles of 2-mercapto ethanol in a total volume of 0·4 ml., was incubated normally for 30 min. at 37° with 0·1 ml. of the enzyme solution containing approx. 0·6 unit. At the end of the incubation 0·3 ml. of bovine serum albumin solution (2 mg./ml.) was added as a carrier and, after mixing, 1·5 ml. of ice-cold N-$HClO_4$. After mixing again the tubes were allowed to stand for 10 min. at 0° before the addition of 0·9 ml. of ice-cold water. The precipitate was removed by centrifugation at 2° at 1000*g* for 15 min. and the extinction of the supernatant liquid at 260 mμ was measured. The amount of DNA rendered acid-soluble was calculated by assuming a molar extinction coefficient at 260 mμ in acid of 10200 (Keir, 1962).

The activity of the liver nuclease on native DNA was measured as above but with 300 μg. of native DNA replacing denatured DNA. The assay medium for deoxyribonuclease II activity contained 300 μg. of native DNA, 25 μmoles of acetate buffer, pH 4·7, and 100 μmoles of KCl in a total volume of 0·4 ml.; otherwise the procedure was as described above.

(*b*) Assay of ribonuclease. The activity of the liver nuclease towards RNA was measured as for deoxyribonuclease except that the denatured DNA was replaced by 300 μg. of RNA. The corresponding molar extinction coefficient is 10600.

(*c*) Assay of phosphomonoesterase and phosphodiesterases I and II. The assay medium contained 2 μmoles of *p*-nitrophenyl phosphate, thymidine 5′-*p*-nitrophenyl phosphate or thymidine 3′-*p*-nitrophenyl phosphate respectively with 25 μmoles of imidazole–HCl buffer, pH 6·8, 0·25 μmole of $MgCl_2$ and 5 μmoles of 2-mercaptoethanol in a volume of 0·4 ml. The enzyme solution was added in a volume of 0·1 ml. and the assay mixture was incubated at 37°. The reaction was stopped by the addition of 2·5 ml. of 0·1 N-NaOH and the extinction at 400 mμ was measured. The molar extinction coefficient under these conditions is 12000 (Razzell & Khorana, 1959).

Determination of protein. Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine serum albumin as a standard. For effluents from columns, the extinction at 280 mμ gave an approximate indication of the distribution of protein.

Determination of ammonium salts. Ammonium salts were estimated with a commercial Nessler's reagent (British Drug Houses Ltd.). The solution was made up to a total volume of 7 ml. with water, 1 ml. of Nessler's reagent was added and the extinction at 490 mμ was measured. This procedure was satisfactory for samples containing 5–100 μg. of nitrogen.

RESULTS

Purification of rat-liver deoxyribonuclease

Preparation of mitochondria. Ten male Porton strain rats from the Department colony weighing 180–220 g. were given water but no food overnight. The rats were anaesthetized with ether and killed by exsanguination. The livers were excised and immersed in crushed ice. Subsequent operations were carried out at 0–4° except where otherwise stated. The livers were weighed and cut into small pieces before homogenization in 8 vol. of 0·25 M-sucrose in a cooled Potter-type homogenizer. After the initial suspension of the tissue, about five passes of the pestle were found to give a homogenate relatively free of whole cells, as judged by microscopic examination of a smear stained with 1% crystal violet in 0·1 M-citric acid. The homogenate was centrifuged at 600*g* for 10 min. The supernatant liquid was decanted from the sediment and centrifuged at 14000*g* for 10 min. The supernatant liquid was discarded and the sediment, consisting of mitochondria and lysosomes, was suspended by gentle homogenization into a volume of 10 mM-tris–hydrochloric acid buffer, pH 7·5, made 0·1% with respect to Triton X-100, 1·25 times the volume of the original homogenate.

Ammonium sulphate fractionation. Saturated ammonium sulphate solution adjusted to pH 7·5 with aqueous ammonia was added slowly with stirring to the solubilized mitochondria to give 35% saturation. The suspension was kept for 20 min. and then centrifuged at 14000*g* for 20 min. The precipitate was occasionally found to form a pellet floating on the surface as well as at the bottom of the tube, and so the supernatant liquid was strained through two layers of muslin. To this solution a further volume of saturated ammonium sulphate was added slowly to bring the degree of saturation to 55%. Stirring was continued for 20 min. and the precipitate was collected by centrifugation at 14000*g* for 20 min. The precipitate was dissolved in the minimum volume of 10 mM-tris–hydrochloric acid buffer, pH 7·5, and the amount of ammonium sulphate present was estimated with Nessler's reagent. The concentration of ammonium sulphate was adjusted to 80 mg./ml.

Acetone fractionation. M-Tris–hydrochloric acid buffer, pH 6·5, was added to the dissolved ammonium sulphate fraction, 2 ml. being added for every 10 ml. of the solution. The solution was then immersed in an ethylene glycol bath at −15° and the measured volume of acetone, previously cooled to −15°, to bring the solution to 36·9% (v/v) acetone was added immediately with stirring. Stirring was continued for 5 min. and the precipitate was then removed by centrifugation at 700*g* for 15 min. at −15°. The supernatant liquid was decanted into a conical flask that had previously been immersed in the ethylene glycol bath at −15°. To it was added the required volume of precooled acetone to bring the concentration of acetone to 46·9% (v/v). The solution was stirred for 5 min.,

after which it was centrifuged at 700*g* for 15min. at −15°. The precipitate formed a protein pellet situated between two liquid phases. Occasionally no such separation of the liquid phases occurred and it was invariably found that in these circumstances the enzyme preparation was relatively inactive. The liquid was carefully drained away, the pellets were dissolved in a minimum volume of 10mM-tris–hydrochloric acid buffer, pH 7·5, and the solution was dialysed against 7l. of 10mM-tris–hydrochloric acid buffer, pH 7·5, for about 5hr. The solution was frozen and stored at −20°. No significant losses of activity occurred after storing for 1–2 months at this stage.

DEAE-cellulose. The bed volume of the column was such that the protein/DEAE-cellulose ratio did not exceed 1mg. of protein/3ml. packed bed volume. The column (4·7cm. × 40cm.) was equilibrated with 10mM-tris–hydrochloric acid buffer, pH 8·0. The acetone fraction was applied to the column and washed through with 10mM-tris–hydrochloric acid buffer, pH 8·0, contained in a reservoir held 1m. above the surface of the cellulose. Under such conditions a flow rate of approx. 700ml./hr. was obtained. Initially it was necessary to measure the protein content and deoxyribonuclease activity of those fractions containing ultraviolet-absorbing material. The fractions containing activity were then combined. However, since the activity and protein were eluted together, it was found sufficient to combine those fractions with an extinction at 280mμ greater than 50% of the maximum extinction obtained (Fig. 1). Though no separation of activity from inactive protein is apparent in Fig. 1, more than 95% of the protein applied to the column was retained and about 30% of the enzymic activity was eluted (Table 1).

Sephadex G-75. The Sephadex G-75 column (1·7cm. × 106cm.) was equilibrated at 0–4° with 10mM-imidazole–hydrochloric acid buffer, pH 6·8, made 5mM with respect to 2-mercaptoethanol. The void volume had previously been determined with Blue Dextran 2000 and found to be 72ml. The enzyme was concentrated from the DEAE-cellulose effluent by precipitation in 70% saturated ammonium sulphate. The precipitate was dissolved in 1ml. of 10mM-imidazole–hydrochloric acid buffer, pH 6·8, made 5mM with respect to 2-mercaptoethanol, and applied to the bed surface of the column. The column was connected to a reservoir containing the same buffer and the flow rate was adjusted to 8ml./hr. by varying the height of the reservoir. Fractions (3ml.) were collected and assayed for protein and deoxyribonuclease (Fig. 2). Those fractions containing the enzyme with a specific activity greater than 50% of the maximum

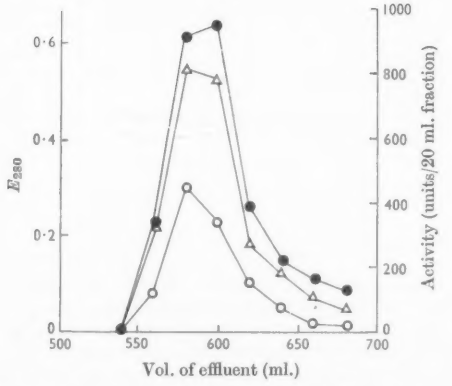

Fig. 1. Elution pattern of the liver nuclease from a column of DEAE-cellulose. The acetone fraction (19ml.) contained 210mg. of protein, 1880 deoxyribonuclease units and 2960 ribonuclease units. The column (4·7cm. × 40cm.) was equilibrated with 10mM-tris–HCl buffer, pH 8·0, and was eluted with the same buffer. The effluent was collected in 20ml. fractions after the first 400ml. The extinction at 280mμ of each fraction was measured, and each fraction was assayed for deoxyribonuclease and ribonuclease (as described in the Experimental section). The total protein recovered was 7mg. ●, Protein; ○, deoxyribonuclease activity; △, ribonuclease activity.

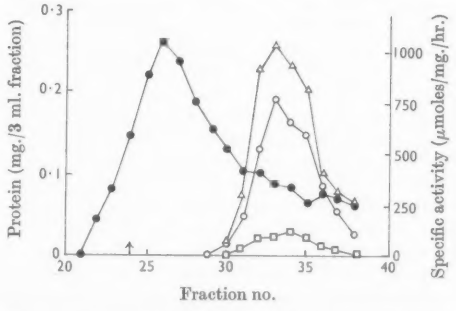

Fig. 2. Fractionation of the liver nuclease on a column of Sephadex G-75. The fraction from DEAE-cellulose was precipitated with $(NH_4)_2SO_4$, dissolved in 1ml. of 10mM-imidazole–HCl buffer, pH 6·8, made 5mM with respect to 2-mercaptoethanol, and contained 4·5mg. of protein and 405 units of nuclease. The column (1·7cm. × 106cm.) was equilibrated and eluted with the same buffer. The flow rate was 8ml./hr. and 3ml. fractions were collected. The arrow (↑) indicates the void volume. The protein content of each fraction was determined and each fraction was assayed for activity towards denatured DNA, native DNA and RNA (as described in the Experimental section). ●, Protein; ○, activity towards denatured DNA; □, activity towards native DNA; △, activity towards RNA.

Table 1. *Purification of the rat-liver nuclease*

Experimental details are given in the text.

Fraction	Total volume (ml.)	Activity (units/ml.)	Total activity (units)	Total protein (mg.)	Specific activity (units/mg. of protein)	Yield (%)	Purification
Homogenate	500	10	5000	7300	0·69	100	1
Triton X-100	650	9·6	6250	2200	2·85	120	4·1
$(NH_4)_2SO_4$ (35–55% satn.)	80	44·5	3560	720	4·78	71	7
Acetone (36·9–46·9%, v/v)	20	165	3300	210	15·7	66	23
DEAE-cellulose	80	11·7	940	7·0	134	19	195
$(NH_4)_2SO_4$ (0–70% satn.)	1	506	506	4·5	112·5	10	163
Sephadex G-75	12	17·1	206	0·44	492	4	715

value were pooled to give the final enzyme solution. The enzyme was in general used immediately, but could be stored at 0° in 20% (w/v) glycerol for up to a week. A summary of a purification procedure is given in Table 1.

Purity of the preparation. The purified enzyme was assayed for deoxyribonuclease II, phosphomonoesterase and phosphodiesterase activities. Deoxyribonuclease (1·7 units) was incubated with the various substrates for 4hr. at 37°. Under these conditions deoxyribonuclease II, phosphomonoesterase and phosphodiesterase individually amounted to no more than 0·1% of the total deoxyribonuclease activity.

The preparation hydrolysed RNA and denatured DNA at comparable rates and native DNA less rapidly. The elution profile on Sephadex G-75 (Fig. 2) of the activity towards RNA and native DNA suggests that the activities belong to the same protein as that hydrolysing denatured DNA. This possibility draws support from the observation that the proportional activities towards RNA, native DNA and denatured DNA of the various fractions obtained during the purification procedure are relatively constant (Table 2), the main discrepancies being in the homogenate and the final fractions. The values of the proportional activities for the homogenate are probably not too meaningful since they represent net results of many concurrent reactions, whereas those shown for the final fractions are rather different from the proportions obtained at earlier stages in the purification and closer values have been obtained in other experiments.

The enzyme hydrolysed s-RNA at a rate such that the ratio of the rate of hydrolysis of denatured DNA to that of native DNA (calf-thymus DNA, purchased from the Sigma Chemical Co.) was approximately the same as the ratio of the rate of hydrolysis of single-stranded RNA to that of s-RNA.

Table 2. *Ratios of the activities of various enzyme fractions towards (i) denatured DNA and native DNA and (ii) RNA and denatured DNA*

Experimental details are given in the text.

Fraction	Activity with denatured DNA / Activity with native DNA	Activity with RNA / Activity with denatured DNA
Homogenate	13·7	3·3
Triton X-100	2·3	2·8
$(NH_4)_2SO_4$ (35–55% satn.)	2·8	3·6
Acetone (36·9–46·9%, v/v)	3·2	2·3
DEAE-cellulose	2·2	3·4
$(NH_4)_2SO_4$ (0–70% satn.)	1·9	4·0
Sephadex G-75 (fractions 32–35)	7·0	1·5

Storage of the nuclease

Before the DEAE-cellulose step in the purification, the enzyme preparation could be stored at −20° for 1–2 months without any loss of activity. After passage through DEAE-cellulose the stability of the enzyme was considerably decreased, so that large losses of activity occurred on storage by freezing or freeze-drying. In the presence of 20% glycerol or 10mM-2-mercaptoethanol the enzyme retained both deoxyribonuclease and ribonuclease activity when stored at 0° for 1 week (Table 3). The assays for deoxyribonuclease and ribonuclease showed no dependency on 2-mercaptoethanol when a freshly prepared DEAE-cellulose effluent was used. However, if this solution was stored in ice for more than 5hr., both assays were stimulated by as much as 17% by 2-mercaptoethanol.

Table 3. *Effect of storage on the activity of the liver nuclease*

Samples (2 ml.) contained 20 mM-imidazole–HCl buffer, pH 6·8, and 83 μg. of protein from the DEAE-cellulose fraction/ml. One sample was stored in ice without further additions, a second sample contained glycerol (final concn. 20%, w/v), and a third was made 10 mM with respect to 2-mercaptoethanol. The samples were assayed for deoxyribonuclease and ribonuclease (as described in the Experimental section) at zero time and after 3, 6 and 28 days. The results are expressed as percentages of the zero-time values.

		Activity (% of original activity)		
Storage conditions	Activity measured	3 days	6 days	28 days
Buffer only	Deoxyribonuclease	3·8	0	4
	Ribonuclease	50·5	35·6	24
20% Glycerol	Deoxyribonuclease	107	111	52
	Ribonuclease	111	111	79
10 mM-Mercaptoethanol	Deoxyribonuclease	49·5	50	65
	Ribonuclease	112	115	85

DISCUSSION

The procedure described for the purification of the liver nuclease provides a means of obtaining the enzyme relatively free of contaminating activities, in particular deoxyribonuclease II, phosphomonoesterase and phosphodiesterase, so that the characteristics and specificity of the enzyme may usefully be examined. The enzyme preparation is not homogeneous with respect to protein, as is clearly indicated by its behaviour on Sephadex G-75. Three pieces of evidence suggest that the nucleolytic activities towards RNA, native DNA and denatured DNA are associated with the same protein. First, the elution profiles of the three activities from a Sephadex G-75 column are exactly superimposed; secondly, the proportional activities of the preparation towards denatured DNA, native DNA and RNA are approximately constant for the fractions obtained during the purification procedures; thirdly, the ratio of the rate of hydrolysis of denatured DNA to that of native DNA is approximately equal to the ratio of the rate of hydrolysis of single-stranded RNA to that of s-RNA.

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Properties of a Purified Rat-Liver Nuclease

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1. The pH optimum, ionic requirement and heat-stability of a purified liver nuclease have been examined with RNA and denatured DNA as substrates. 2. The enzyme attacked DNA and RNA in an endonucleolytic manner, forming oligonucleotides terminated by 5′-phosphate groups. No clear specificity was found with respect to the bases at the site of cleavage. 3. Comparison of the results obtained with RNA and denatured DNA as substrates suggests that the ribonuclease and deoxyribonuclease activities are associated with the same protein.

The characteristics of the nuclease purified from rat-liver mitochondria (see Curtis, Burdon & Smellie, 1966) have been examined to determine whether it could be identified with a known nuclease in rat liver, e.g. the acid ribonuclease and deoxyribonuclease of the lysosomes (Roth, 1957), the alkaline ribonuclease (ribonuclease I) of the nuclei (Heppel, Ortiz & Ochoa, 1956) or the alkaline ribonuclease (ribonuclease II) of the mitochondria (Roth, 1957).

MATERIALS AND METHODS

Materials. Salmon-sperm DNA, adenosine, guanosine, cytidine and adenosine 2′- and 3′-monophosphate were purchased from the California Corp. for Biochemical Research, Lucerne, Switzerland, inosine from the Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A., deoxyinosine and thymidine from the Sigma Chemical Co., London, S.W. 6, and deoxyadenosine, deoxyguanosine, deoxycytidine and adenosine 5′-monophosphate from Schwarz BioResearch Inc., Mount Vernon, N.Y., U.S.A.

Pancreatic deoxyribonuclease I, pancreatic ribonuclease, spleen phosphodiesterase and crude snake venom (*Crotalus adamanteus*) were purchased from the Sigma Chemical Co., and *Escherichia coli* alkaline phosphatase as an $(NH_4)_2SO_4$ suspension from the Nutritional Biochemicals Corp. Purified snake-venom phosphodiesterase was prepared from the crude venom by a combination of published methods as follows: step 1, acetone fractionation (Bjork, 1963); step 2, ethanol fractionation (Williams, Sung & Laskowski, 1961); step 3, ethanol fractionation (Williams *et al.* 1961); step 4, acetone fractionation (Sulkowski & Laskowski, 1962); step 5, CM-cellulose (Felix, Potter & Laskowski, 1960); step 6, DEAE-cellulose (Felix *et al.* 1960). The spleen and purified snake-venom phosphodiesterases showed no detectable phosphomonoesterase activity after incubation at 37° for 4 hr. with 2′- and 3′-AMP and 5′-AMP respectively when the products of the digestion were examined chromatographically.

Liver nuclease. Unless otherwise stated the pooled fraction of the liver nuclease from Sephadex G-75 was used. The methods employed for assay of the activity of this enzyme towards native DNA, denatured DNA and RNA were as described by Curtis *et al.* (1966).

Inactivation of E. coli *alkaline phosphatase.* The solution was refluxed with an equal volume of ethanol for 1 hr. after which the ethanol was allowed to evaporate. An equal volume of chloroform was added and the solution was shaken vigorously for 2 hr., after which the two layers were separated by centrifuging.

Column chromatography of oligonucleotides. A DEAE-cellulose column (2 cm. × 40 cm.) equilibrated with 10 mM-triethylammonium hydrogen carbonate buffer, pH 8·6, was used. The column was developed by using a linear gradient with 1·4 l. of 10 mM-triethylammonium hydrogen carbonate buffer, pH 8·6, in the mixing vessel and 1·4 l. of 1·0 M-triethylammonium hydrogen carbonate buffer, pH 8·6, in the reservoir. A hydrostatic pressure of 1 m. above the top of the column was used. The gradient was measured by determining the nitrogen content of selected fractions. Samples were digested with conc. H_2SO_4 and the resulting $(NH_4)SO_4$ was estimated, after neutralization with alkali, with Nessler's reagent.

Paper chromatography. For the separation of deoxyribonucleosides a two-solvent system described by Sulkowski & Laskowski (1962) was used. Solvent I consisted of 95% (v/v) ethanol–M-ammonium acetate adjusted to pH 7·5 (2:1, v/v) and solvent II was propan-2-ol–water–satd. $(NH_4)_2SO_4$ (1:9:40, by vol.). Whatman no. 3MM paper was used and both solvents were used in a descending direction. The development time for the first solvent was 16 hr. and for the second 12 hr. The ribonucleosides separated in a similar manner except that cytidine and uridine overlapped. The proportion of these pyrimidines in the merging spots was estimated by comparing the spectra of the eluted spots with the spectra of known mixtures of cytidine and uridine.

Solvent III was propan-2-ol–water–aq. NH_3 (sp.gr. 0·88) (7:2:1, by vol.). Whatman no. 1 paper was used with this solvent and the development time was 16 hr.

Spots were located by viewing the chromatograms with a Hanovia Chromatolite lamp. The material in a spot was eluted by cutting out the spot and attaching it to a paper

wick, which was then immersed in 10mM-HCl. The eluted material was identified and estimated by recording the spectrum in a Cary model 11 spectrophotometer and by utilizing the data given in the publication *Properties of the Nucleic Acid Derivatives* by Calbiochem, Los Angeles, Calif., U.S.A.

Examination of the products of prolonged digestion. The digestion mixture contained 40mg. of heat-denatured DNA from Landschutz ascites-tumour cells, 1·5m-moles of imidazole–HCl buffer, pH6·8, 15μmoles of $MgCl_2$ and 300μmoles of 2-mercaptoethanol in a total volume of 20ml. The solution was incubated at 37° for 12hr. and at zero time 90 deoxyribonuclease units of liver nuclease were added followed by a further 90 units after 4hr. and 8hr. After incubation the solution was deproteinized by shaking with an equal volume of chloroform–3-methylbutan-1-ol (4:1, v/v) for 1hr. The aqueous layer was diluted to 150ml. and applied to a DEAE-cellulose column (2cm.×40cm.), after which gradient elution was commenced. The effluent was divided into fractions according to the E_{260} values. The pooled fractions were freeze-dried until the characteristic smell of triethylamine could no longer be detected. The material was then dissolved in 4ml. of water.

The chain length of the pooled fractions was determined by comparing the total phosphorus with the terminal phosphorus of a sample of an oligonucleotide fraction. The terminal phosphorus was estimated by incubating the sample with 0·02ml. of alkaline-phosphatase suspension and 50μmoles of tris–HCl buffer, pH8·0, in a volume of 2ml. at 37° for 1hr. The inorganic phosphorus released was estimated by the method of Allen (1940).

The digestion products of the ribonuclease activity on 40mg. of yeast RNA were examined in a similar manner.

Position of the terminal phosphate. Dephosphorylated oligodeoxyribonucleotides were prepared by the digestion of 4mg. of DNA from Landschutz ascites-tumour cells with 200μg. of pancreatic deoxyribonuclease in the presence of 200μmoles of tris–HCl buffer, pH7·5, and 100μmoles of $MgCl_2$ in a total volume of 5ml. at 37° for 1hr. The pH of the solution was then adjusted to 8·0 and 0·01ml. of alkaline-phosphatase suspension was added. The solution was incubated at 37° for a further 1hr. The phosphatase was then inactivated. Dephosphorylated oligoribonucleotides were prepared similarly by the digestion of 4mg. of yeast RNA with 200μg. of pancreatic ribonuclease.

Oligodeoxyribonucleotides were also prepared by incubating 2mg. of denatured salmon-sperm DNA with 25μmoles of imidazole–HCl buffer, pH6·8, 0·5μmole of $MgCl_2$, 10μmoles of 2-mercaptoethanol and 5 deoxyribonuclease units of the purified liver nuclease at 37° for 4hr. Oligoribonucleotides were similarly prepared from 2mg. of yeast RNA.

The solutions of the dephosphorylated oligodeoxyribonucleotides prepared from the deoxyribonuclease I digest and the oligodeoxyribonucleotide products of the liver nuclease were each divided into three fractions. The first fraction gave a measure of mononucleotides released in the preliminary digestion. The second fraction was incubated at pH6·5 and 37° for 1hr. after adding 200μg. of spleen phosphodiesterase. The reaction was stopped by heating at 100° for 10min. The pH of the third fraction was adjusted to pH8·8 and 0·1ml. of the purified snake-venom phosphodiesterase was added. Incubation was at 37° for 1hr. and then the reaction was stopped by heating at 100° for 10min. The dephosphorylated oligoribonucleotides from the pancreatic-ribonuclease digest and the oligoribonucleotide products of the liver nuclease were treated similarly. To facilitate the chromatographic separation of the mononucleotides produced by the action of the phosphodiesterase from the other products each fraction was dephosphorylated. The pH of each fraction was adjusted to 8·0, 0·01ml. of alkaline-phosphatase suspension was added and the solutions were incubated at 37° for 30min. The fractions were concentrated by freeze-drying and the material was redissolved in 0·05ml. of 10mM-HCl. Then 0·03ml. of each fraction was spotted on Whatman no. 1 paper and the chromatogram was developed in a descending direction in solvent III for 16hr.

Determination of the terminal bases of the oligonucleotides. The oligodeoxyribonucleotides and oligoribonucleotides were prepared by incubating 20mg. of salmon-sperm DNA or 20mg. of yeast RNA, 250μmoles of imidazole–HCl buffer, pH6·8, 5μmoles of $MgCl_2$, 100μmoles of 2-mercaptoethanol and 50 units of the liver nuclease in a total volume of 10ml. at 37° for 4hr. The two solutions of oligonucleotides were dephosphorylated by adjusting the pH of each solution to 8·0 and adding 0·02ml. of alkaline-phosphatase suspension. The solutions were incubated at 37° for 1hr., the alkaline phosphatase was inactivated and the two solutions were divided into two. One half was adjusted to pH8·8, 0·5ml. of purified snake-venom phosphodiesterase was added and the solution was incubated at 37° for 4hr. In this time the digestion had proceeded to completion. The other half was adjusted to pH6·5, 1·0mg. of spleen phosphodiesterase was added and the solution was incubated at 37° for 8hr., the extent of the digestion being approx. 90%. The four digests were freeze-dried and redissolved in 0·1ml. of 10mM-HCl. The nucleoside and nucleotide products were separated by the two-dimensional paper-chromatographic system of Sulkowski & Laskowski (1962).

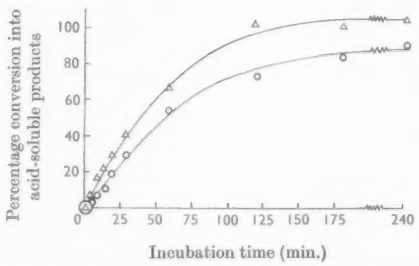

Fig. 1. Time-course of the hydrolysis of RNA and denatured DNA by the purified liver nuclease. The assay medium contained 200μg. of RNA or denatured DNA, 25μmoles of imidazole–HCl buffer, pH6·8, 0·25μmole of $MgCl_2$, 5μmoles of 2-mercaptoethanol and 6·7μg. of protein from the DEAE-cellulose fraction for deoxyribonuclease assays or 2·7μg. for ribonuclease assays in a total volume of 0·5ml. Incubation was at 37° for the indicated time-intervals. The results are expressed as percentages of the amount of the substrate converted into acid-soluble products. ○, Deoxyribonuclease activity; △, ribonuclease activity.

RESULTS

Since the rate of hydrolysis of RNA was greater than that of DNA, less enzyme was employed in experiments with RNA. The time-course of the hydrolysis of denatured DNA and RNA showed that the rate was linear for approx. 1 hr., when 50% of the substrate had become acid-soluble (Fig. 1). With RNA as a substrate there was direct proportionality between the rate of hydrolysis and the concentration of the enzyme over the range 0–10 μg. of protein/ml. (Fig. 2). The rate of hydrolysis of DNA showed lack of proportionality at concentrations below 12 μg. of protein/ml. (Fig. 2). Above this concentration there was direct proportionality.

The effect of pH was studied in a number of different buffer systems. It was found that phosphate and glycine–sodium hydroxide buffers gave relatively low activities in comparison with imidazole–hydrochloric acid and tris–hydrochloric acid buffers. Both activities showed sharp pH optima, deoxyribonuclease at pH 6·6–6·8 and ribonuclease at pH 6·8–7·0 (Fig. 3).

The two activities showed an absolute requirement for Mg^{2+} or Mn^{2+} (Fig. 4). The requirement was about the same for both cations and for both activities, i.e. the optimum concentration for deoxyribonuclease and ribonuclease was about 0·1 mM with Mg^{2+} and with Mn^{2+}. In the absence of Mg^{2+}, Ca^{2+} showed no stimulatory effect (Fig. 5), and inhibited both activities in the presence of Mg^{2+}. There was no indication of synergism in the presence of both cations. Univalent cations inhibited both activities markedly, though at a higher concentration than Ca^{2+} (Fig. 6).

The effect of heat was examined at pH 5·1 and pH 7·2 (Fig. 7). Both deoxyribonuclease and ribonuclease were rapidly inactivated at pH 5·1, but at pH 7·2 both activities survived heating at higher temperatures than at pH 5·1.

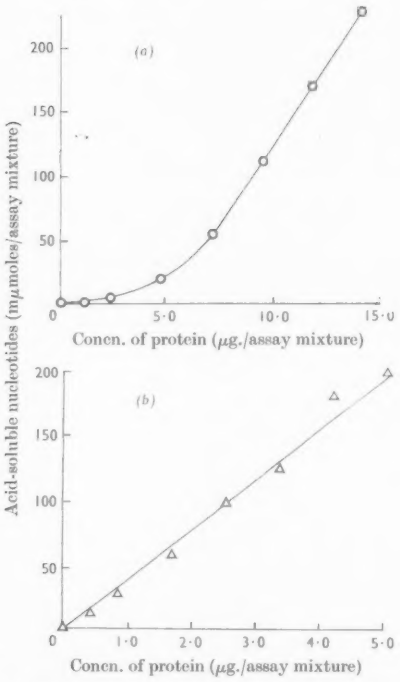

Fig. 2. Effect of the protein concentration on the rate of hydrolysis of RNA and denatured DNA. The assay medium contained 300 μg. of RNA or denatured DNA, 25 μmoles of imidazole–HCl buffer, pH 6·8, 0·25 μmole of $MgCl_2$, 5 μmoles of 2-mercaptoethanol and various amounts of protein of the DEAE-cellulose fraction in a total volume of 0·5 ml. Incubation was at 37° for 30 min. (*a*) ○, Deoxyribonuclease activity; (*b*) △, ribonuclease activity.

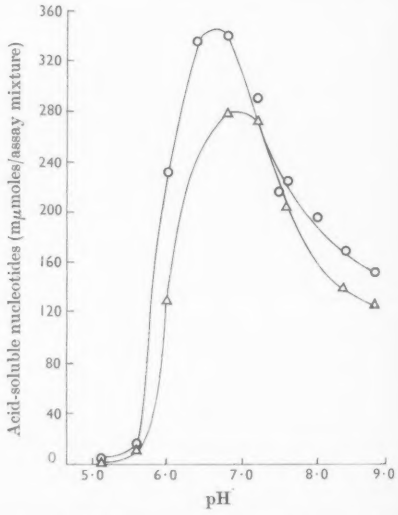

Fig. 3. Effect of pH on the activity of the purified liver nuclease. The assay medium contained 300 μg. of RNA or denatured DNA, 0·25 μmole of $MgCl_2$, 5 μmoles of 2-mercaptoethanol, 25 μmoles of buffer and 1·1 μg. of purified liver nuclease protein for deoxyribonuclease assays or 0·6 μg. for ribonuclease assays in a total volume of 0·5 ml. The buffers used were: (i) acetate at pH 5·1 and 5·6; (ii) imidazole–HCl at pH 6·0–7·6; (iii) tris–HCl at pH 7·5–8·8. Incubation was at 37° for 30 min. ○, Deoxyribonuclease activity; △, ribonuclease activity.

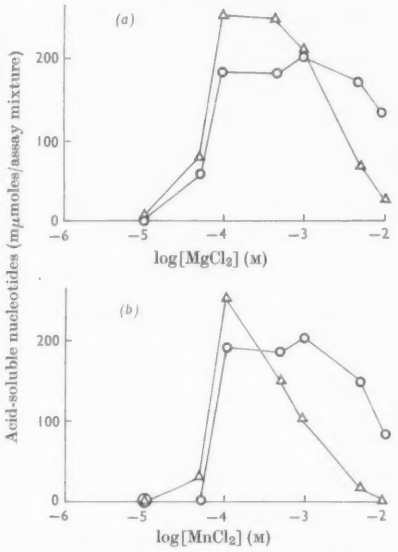

Fig. 4. Effect of Mg^{2+} and Mn^{2+} on the activity of the purified liver nuclease. The assay medium contained 300μg. of RNA or denatured DNA, 25μmoles of imidazole–HCl buffer, pH 6·8, 5μmoles of 2-mercaptoethanol and 0·9μg. of purified liver nuclease protein for deoxyribonuclease assays or 0·45μg. for ribonuclease assays in a total volume of 0·5 ml. $MgCl_2$ (*a*) and $MnCl_2$ (*b*) were added to give the indicated molarities. Incubation was at 37° for 30 min. ○, Deoxyribonuclease activity; △, ribonuclease activity.

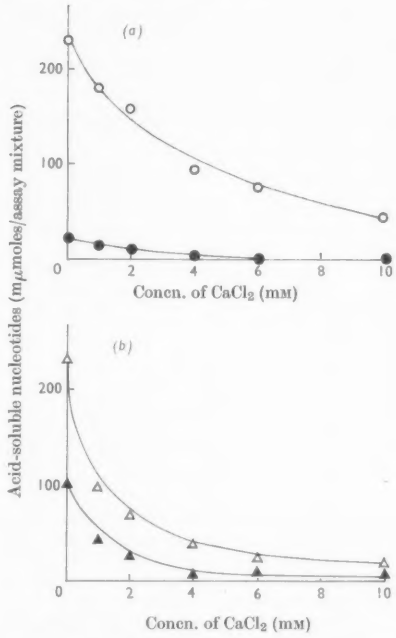

Fig. 5. Effect of $CaCl_2$ in the presence and absence of the optimum concentration of $MgCl_2$ on the activity of the purified liver nuclease. The assay medium contained 300μg. of RNA or denatured DNA, 25μmoles of 10 mM-imidazole–HCl buffer, pH 6·8, 5μmoles of 2-mercaptoethanol and 0·9μg. of purified liver nuclease protein for deoxyribonuclease assays or 0·45μg. for ribonuclease assays in a total volume of 0·5 ml. Incubation was at 37° for 30 min. (*a*) Deoxyribonuclease activity in the absence (●) and presence (○) of 0·25μmole of $MgCl_2$; (*b*) ribonuclease activity in the absence (▲) and presence (△) of 0·25μmole of $MgCl_2$.

Specificity of the liver nuclease

Products of prolonged digestion. Exhaustive digestion of the substrate was excluded since it was considered that under conditions necessary to achieve complete hydrolysis of the substrate the contaminating activities, i.e. phosphomonoesterase and phosphodiesterase, would contribute appreciably to the result. A 12 hr. incubation was therefore used since after such a time exo- and endonucleolytic activity would be clearly distinguished. The elution profiles of the DNA and RNA digests on DEAE-cellulose are given in Figs. 8 and 9: 98% of the ultraviolet-absorbing material of the DNA digest and 100% of that in the RNA digest were recovered. The chain lengths of the fractions indicated in Figs. 8 and 9 were determined as described in the Materials and Methods section (Table 1): for both DNA and RNA about 95% of the recovered nucleotide was in the form of oligonucleotides. To enable these results to be evaluated the phosphomonoesterase and phosphodiesterase activities in the enzyme solutions used in both digests were assayed and the values obtained were calculated so that the extent of hydrolysis due to the contaminating activities under the conditions of the digestion could be estimated. It was found that for the DNA digest phosphomonoesterase would account for 0·3% and phosphodiesterase II for 0·25% of the total digest, and phosphodiesterase I showed no activity. For the RNA digest phosphomonoesterase would account for 0·18% and phosphodiesterases I and II for 0·02% each of the total digest.

The results indicate that both activities, deoxyribonuclease and ribonuclease, are almost exclu-

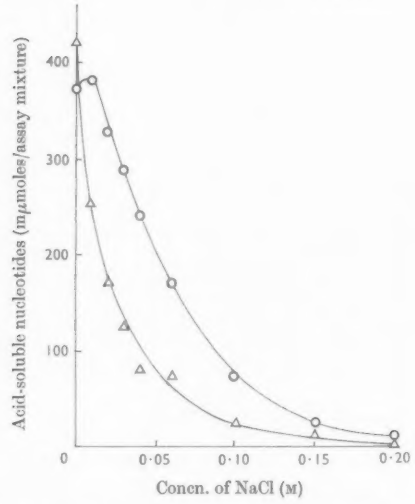

Fig. 6. Effect of NaCl on the activity of the purified liver nuclease. The assay medium contained 300 μg. of RNA or denatured DNA, 25 μmoles of imidazole–HCl buffer, pH 6·8, 0·25 μmole of $MgCl_2$, 5 μmoles of 2-mercaptoethanol and 3 μg. of purified liver nuclease protein for deoxyribonuclease assays or 1·5 μg. for ribonuclease assays in a total volume of 0·5 ml. NaCl was added to give the indicated molarities. Incubation was at 37° for 30 min. ○, Deoxyribonuclease activity; △, ribonuclease activity.

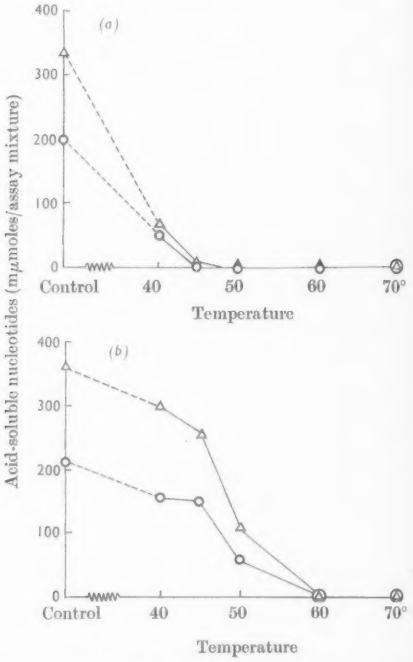

Fig. 7. Effect of heat on the activity of the purified liver nuclease. Samples containing 11 μg. of purified liver nuclease protein and 50 μmoles of acetate buffer, pH 5·1, or imidazole–HCl buffer, pH 7·2, in a volume of 1·2 ml. were heated at 40°, 45°, 50°, 60° or 70° for 5 min. and then were immediately cooled in ice until assayed. The control was kept in ice until assayed. The assay medium contained 300 μg. of RNA or denatured DNA, 25 μmoles of imidazole–HCl buffer, pH 6·8, 0·25 μmole of $MgCl_2$, 5 μmoles of 2-mercaptoethanol and 0·1 ml. of the samples in a total volume of 0·5 ml. Incubation was at 37° for 30 min. (*a*) Heated at pH 5·1; (*b*) heated at pH 7·2. ○, Deoxyribonuclease activity; △, ribonuclease activity.

sively endonucleolytic in their mode of action, though the amount of mononucleotide product cannot be accounted for by phosphodiesterase activity.

Determination of the position of the terminal phosphate group. The position of the terminal phosphate group was determined by comparing the hydrolysis by snake-venom and spleen phosphodiesterases of dephosphorylated oligodeoxyribonucleotides and oligoribonucleotides with the hydrolysis of oligonucleotides produced by the liver nuclease. The extent of hydrolysis by the phosphodiesterases was examined by paper chromatography. The mononucleotides were hydrolysed to nucleosides to facilitate their complete separation from all other products. The material corresponding in R_F to the four known nucleosides was eluted and the E_{260} value measured (Table 2). The results show that, by comparison with the dephosphorylated oligonucleotides, the oligodeoxyribonucleotides and oligoribonucleotides were both relatively resistant to spleen phosphodiesterase but both were readily hydrolysed by snake-venom phosphodiesterase. Since snake-venom phosphodiesterase attacks oligonucleotides bearing terminal 5′-phosphate but not terminal 3′-phosphate groups, and spleen phosphodiesterase shows the reverse specificity, it was concluded that the deoxyribonuclease and ribonuclease activities of the purified liver nuclease produce oligonucleotides terminated by 5′-phosphate groups.

Determination of the terminal bases of the oligonucleotides. Dephosphorylated oligonucleotides were produced by hydrolysis of DNA and RNA by the purified liver nuclease followed by incubation with alkaline phosphatase. After inactivation of the alkaline phosphatase, hydrolysis of the

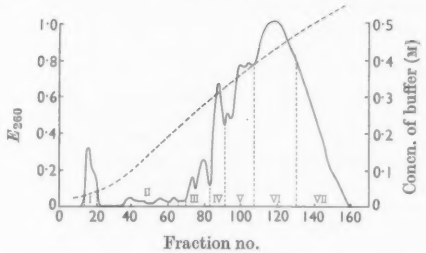

Fig. 8. Fractionation of the products of digestion of denatured DNA by the purified liver nuclease. The digestion medium contained 40mg. of denatured Landschutz ascites-carcinoma DNA, 1·5m-moles of imidazole–HCl buffer, pH6·8, 15μmoles of $MgCl_2$ and 300μmoles of 2-mercaptoethanol in a total volume of 20ml. and was incubated at 37° for 12hr. At zero time 90 deoxyribonuclease units of the purified liver nuclease were added and again after 4hr. and 8hr. The gradient, consisting of 1·4l. of 10mM-triethylammonium hydrogen carbonate buffer, pH8·6, and 1·4l. of 1·0M-trimethylammonium hydrogen carbonate buffer, pH8·6, was applied to a column (2cm.×40cm.) of DEAE-cellulose. The flow rate was 4ml./min. and 16·5ml. fractions were collected. ——, E_{260}; ----, concn. of buffer.

Table 1. *Chain length of the products of digestion of RNA and denatured DNA by liver nuclease*

Experimental details are given in the text.

Fraction		Chain length (no. of nucleotides)	% of total digest
RNA			
I	(18–31)	0	5·7
II	(36–60)	1*	2·9
III	(63–72)	1·9	3·4
IV	(73–96)	2·5	13·1
V	(97–114)	2·8	21·9
VI	(115–123)	2·9	13·1
VII	(124–134)	3·4	16·0
VIII	(135–170)	5·3	24·0
DNA			
I	(15–21)	0	3·3
II	(37–66)	1*	1·8
III	(70–83)	2·2	4·3
IV	(84–91)	2·7	7·2
V	(92–107)	4·7	18·9
VI	(108–130)	6·1	43·8
VII	(131–160)	10·1	20·7

* The position of this fraction in the DEAE-cellulose effluent and the presence of phosphorus in the H_2SO_4 digest indicate that these fractions contain mononucleotides, though no phosphorus was released by alkaline phosphatase.

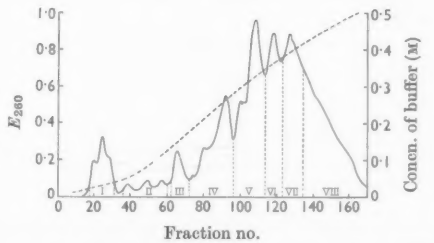

Fig. 9. Fractionation of the products of digestion of RNA by the purified liver nuclease. The digestion medium contained 40mg. of yeast RNA, 1·5m-moles of imidazole–HCl buffer, pH6·8, 15μmoles of $MgCl_2$ and 300μmoles of 2-mercaptoethanol in a total volume of 20ml. and was incubated at 37° for 12hr. At zero time 90 ribonuclease units of the purified liver nuclease were added and again after 4hr. and 8hr. The gradient, consisting of 1·4l. of 10mM-triethylammonium hydrogen carbonate buffer, pH8·6, and 1·4l. of 1·0M-trimethylammonium hydrogen carbonate buffer, pH8·6, was applied to a column (2cm.×40cm.) of DEAE-cellulose. The flow rate was 4ml./min. and 16·5ml. fractions were collected. ——, E_{260}; ----, concn. of buffer.

dephosphorylated oligonucleotides with snake-venom phosphodiesterase released the terminal bases at the 5′-hydroxy end as nucleosides. Similarly, hydrolysis with spleen phosphodiesterase released the terminal bases at the 3′-hydroxy end as nucleosides.

The nucleosides were resolved by two-dimensional paper chromatography with the solvent systems of Sulkowski & Laskowski (1962). The digests gave one ultraviolet-absorbing spot common to all four that ran with the front of solvent I. This spot was identified as 2-mercaptoethanol. Apart from this spot, snake-venom phosphodiesterase gave four spots corresponding in their positions to adenosine, cytosine, guanosine and uridine and to the corresponding deoxy compounds. Digestion with spleen phosphodiesterase produced five spots corresponding in their positions to adenosine, cytosine, guanosine, uridine and inosine and the five corresponding deoxy compounds. The additional spots corresponding to inosine and deoxyinosine were expected, since Winter & Bernheimer (1964) had shown that the commercial preparation of spleen phosphodiesterase contained a deaminase acting on adenosine and deoxyadenosine. Incubation of adenosine and deoxyadenosine with the preparation of spleen phosphodiesterase used in these experiments produced inosine and deoxyinosine.

The nucleosides were eluted and estimated by recording their spectra and utilizing the molar extinction coefficients quoted in the publication

Table 2. *Digestion of the oligonucleotide products of the liver nuclease with spleen and snake-venom phosphodiesterases*

Experimental details are given in the text.

Fraction	E_{260} of nucleotides released: Spleen phosphodiesterase	E_{260} of nucleotides released: Snake-venom phosphodiesterase
DNA products		
(1) Dephosphorylated oligonucleotides	0·44	2·16
(2) Oligonucleotide products of liver nuclease	0·01	1·01
RNA products		
(1) Dephosphorylated oligonucleotides	0·34	0·49
(2) Oligonucleotide products of liver nuclease	0·08	0·64

Table 3. *Terminal nucleosides of the oligonucleotide products of the liver nuclease*

Experimental details are given in the text. The oligonucleotide products are expressed as percentages of the total amount of nucleosides released. The values for adenosine and deoxyadenosine are the sums of adenosine plus inosine and of deoxyadenosine plus deoxyinosine respectively. Values for yeast RNA were taken from Magasanik (1955), and those for salmon-sperm DNA from Chargaff (1955).

Nucleoside	% of total nucleoside at 5′-position	% of total nucleoside at 3′-position	Composition of substrate
Ribonuclease			Yeast RNA
Adenosine	36	46·5	25·0
Cytidine	9·5	8·5	20·1
Guanosine	33·5	30·5	30·2
Uridine	22	15	24·6
Deoxyribonuclease			Salmon-sperm DNA
Deoxyadenosine	30	42	29
Deoxycytidine	17	27	20·8
Deoxyguanosine	25	14	20·4
Thymidine	28	15	29·1

Properties of the Nucleic Acid Derivatives by Calbiochem. The results (Table 3) show that the two activities, deoxyribonuclease and ribonuclease, have no marked preference towards the bases, though when compared with the values for the percentage composition of salmon-sperm DNA and yeast RNA there is a slight predominance of adenine at the 3′-hydroxy end. In the oligoribonucleotides, cytidine occupied the 3′- and 5′-terminal positions less frequently than might have been expected.

DISCUSSION

From the evidence presented in this and the preceding paper (Curtis *et al.* 1966) the enzyme, purified from rat-liver mitochondria, appears to hydrolyse both DNA and RNA. The two activities of the enzyme are very similar in every respect except in their relative stability on storage (see Curtis *et al.* 1966). Thus the enzyme hydrolyses DNA and RNA endonucleolytically producing oligonucleotides terminated by 5′-phosphate groups. It has a preference towards single-stranded rather than double-stranded DNA or RNA, but shows no marked preference towards any of the four bases. The pH optimum for both activities is about 6·8, and the enzyme shows an absolute requirement for Mg^{2+} or Mn^{2+}; Ca^{2+} inhibits the enzyme, as also does Na^{+}. The enzyme is readily denatured by heating, the two activities being lost at equivalent rates.

The deoxyribonuclease activity of the liver nuclease is similar in many respects to pancreatic deoxyribonuclease. The pH optima are similar and the requirement for Mg^{2+} is of the same order; Na^{+} inhibits at approx. 50mM and the products of hydrolysis are similar. However, there are a few important differences. Thus Ca^{2+} alone stimulates pancreatic deoxyribonuclease weakly, but in the presence of Mg^{2+} there is a powerful synergistic

effect (Wiberg, 1958). In contrast, the liver nuclease is inhibited by Ca^{2+} in the presence and absence of Mg^{2+}. The second important difference is that pancreatic deoxyribonuclease hydrolyses native DNA several times more rapidly than denatured DNA, whereas the liver nuclease shows a preference for denatured DNA. In this respect there is a closer similarity to the endonuclease from lamb brain (Healy, Stollar, Simon & Levine, 1963), though this enzyme shows an absolute requirement for denatured DNA. However, the liver nuclease and the lamb-brain enzyme respond to heat, freezing and freeze-drying similarly, and 2-mercaptoethanol and glycerol stabilize both enzymes when they are stored at 0°. In addition, the purified lamb-brain endonuclease contains ribonuclease activity (M. G. Burdon, personal communication), though whether this activity belongs to the same protein as the deoxyribonuclease activity has yet to be determined.

The ribonuclease activity of the liver nuclease is distinct from the enzyme purified from rat-liver mitochondria by Roth (1957) and Beard & Razzell (1964). This ribonuclease has a pH optimum about 7·0, but no requirement for Mg^{2+}; for optimum activity it requires an ionic strength of 0·1 M. It is stable to heat and produces on hydrolysis of RNA a resistant 'core' and oligonucleotides terminated by pyrimidine 2′,3′-(cyclic)-phosphates. Heppel *et al.* (1956) have described an enzyme from guinea-pig nuclei that hydrolyses polyadenylic acid and polyuridylic acid to oligonucleotides terminated by a 5′-phosphate group.

We thank Professor J. N. Davidson, F.R.S., for his interest and encouragement during this work and Dr M. G. Burdon for many valuable discussions. The investigation was aided by grants from the British Empire Cancer Campaign and the Rankine Fund of the University of Glasgow which are gratefully acknowledged. P. J. C. was a Medical Research Council Scholar while these studies were in progress.

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Factors Controlling Amino Acid Incorporation by Ribosomes from Krebs II Mouse Ascites-Tumour Cells

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1. A ribosome–cell sap system capable of supporting the incorporation of ^{14}C-labelled amino acids into protein has been prepared from Krebs II mouse ascites-tumour cells. The requirements of this system for optimum activity and response to added messenger RNA have been investigated. One such system has been obtained for which amino acid incorporation is almost wholly dependent on the addition of suitable messenger RNA. 2. Ribosomes of widely different but predictable activities in the cell-free system have been prepared from Krebs cells pretreated in a variety of ways. The factors in the pretreatment of the cells responsible for these differences have been investigated. 3. The structural and functional properties of these different ribosome preparations and their response to exogenous messenger RNA have been examined and are discussed in the light of modern concepts of the control of protein synthesis.

Previous reports from this Laboratory have described the amino acid-incorporation activity of microsome–cell sap systems obtained from normal and EMC-virus†-infected Krebs II mouse ascites-tumour cells (Krebs cells). It was shown that microsomes from infected cells had a lowered intrinsic activity but that this activity increased markedly in response to added EMC-virus RNA. These experiments were taken to indicate that the m-RNA content of the microsome fraction of infected cells was below normal and that the EMC-virus RNA was capable of functioning as a replacement messenger (Kerr, Martin, Hamilton & Work, 1962). These systems have now been further refined and a ribosome–cell sap preparation has been obtained for which amino acid incorporation is almost wholly dependent on the addition of EMC-virus RNA or polyU.

During this work it was observed that the total amino acid incorporation supported by ribosome–cell sap systems from uninfected cells varied greatly according to the mode of pretreatment of the cells. Further investigation showed that these variations resulted from changes in the ribosome fraction and that, by suitably pretreating cells, ribosomes could be prepared with a variety of predictable activities. Thus ribosomes from cells that, on isolation from mice, were rapidly washed free of ascitic fluid at 0° were 2–3 times as active as those from cells washed slowly or at room temperature. Incubation in a simple buffered salts medium at 37° of the cells washed at room temperature yielded ribosomes of very low activity. When, however, the preincubation was carried out in the presence of glucose, the activity of the ribosomes increased very rapidly until it reached the level of preparations from fresh cold-washed cells.

We have investigated the factors controlling this variation in ribosomal activity and the response of these different ribosome preparations to EMC-virus RNA. Our results suggest that the rate of protein synthesis in mammalian cells may be controlled by factors in addition to those concerned with the rate of m-RNA synthesis.

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† Abbreviations: EMC virus, encephalomyocarditis virus; m-RNA, messenger RNA; s-RNA, 'soluble' or transfer RNA; TBS, tris-buffered saline; PBS, phosphate-buffered saline; polyU, polyuridylic acid.

MATERIALS AND METHODS

Materials. ATP, GTP and pyruvate kinase were obtained from C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany). For use in the cell-free system ATP and GTP solutions of the required molarity were adjusted to pH 6·5–7·0 with N-KOH. Phosphoenolpyruvate, supplied as the silver–barium salt (C. F. Boehringer und Soehne G.m.b.H.), was converted into the potassium salt, and solutions of the required molarity were adjusted to pH 6·5–7·5 with N-KOH, the total concentration of K^+ in the solution being taken into account in the amino acid-incorporation assay. β-Mercaptoethanol (L. Light and Co. Ltd., Colnbrook, Bucks.) was redistilled and made up as a 0·5M solution with

deionized water and adjusted to pH6–7 with N-KOH. These solutions were stored at −18°. Actinomycin D was obtained from Merck and Co. Inc. (Rahway, N.J., U.S.A.), polyU from Miles Chemical Corp. (Elkhart, Ind., U.S.A.), uniformly ^{14}C-labelled amino acids and [1-^{14}C]leucine from The Radiochemical Centre (Amersham, Bucks.) and crystalline pancreatic deoxyribonuclease from the Worthington Biochemical Corp. (Freehold, N.J., U.S.A.).

Buffers and solutions. TBS contained the following (final concentrations): NaCl (146mM); tris buffer (acetate or chloride), pH7·8 (measured at room temperature) (35mM). PBS contained the following (final concentrations): NaCl (136mM); KCl (2·7mM); $MgSO_4$ (0·5mM); $CaCl_2$ (0·9mM); KH_2PO_4 (1·5mM); Na_2HPO_4 (8mM).

Earle's medium was prepared as described by Martin, Malec, Sved & Work (1961) and contained the following (final concentrations): NaCl (117mM); KCl (5·38mM); $MgSO_4$ (0·8mM); $CaCl_2$ (1·8mM); NaH_2PO_4 (1·2mM); $NaHCO_3$ (equilibrated with CO_2 gas) (26mM); glucose (11mM; 0·2%); benzylpenicillin (100i.u./ml.) and streptomycin–$CaCl_2$ (60μg./ml.) (Glaxo Laboratories Ltd., Greenford, Middlesex).

Pseudo-Earle's medium was prepared as for Earle's medium, and contained (final concentrations): NaCl (100mM); KCl (3·7mM); $CaCl_2$ (1·8mM); $MgSO_4$ (0·8mM); Na_2HPO_4 (8mM); KH_2PO_4 (1·5mM); $NaHCO_3$ (equilibrated with CO_2 gas) (26mM); glucose (11mM; 0·2%).

The following solutions were used in the preparation of the ribosome and cell-sap fractions from Krebs cells. Solution A contained (final concentrations): tris buffer, pH7·7–7·8 (210mM); KCl (150mM); β-mercaptoethanol (60mM); EDTA (6mM); magnesium acetate (6mM); sucrose (1·5M). Solution B was made by the addition of 5vol. of 6mM-magnesium acetate to 1vol. of solution A. Solution C contained (final concentrations): tris buffer, pH7·7–7·8 (20mM); β-mercaptoethanol (5mM); EDTA (1mM).

All solutions of EDTA (disodium salt) (Hopkin and Williams Ltd., Chadwell Heath, Essex) were adjusted to pH7·0 with KOH before use.

Estimation of protein. The protein content of ribosome and cell-sap fractions was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951), with crystalline bovine serum albumin as standard.

Propagation of Krebs cells. The origin of the cell line used and its mode of propagation intraperitoneally in mice was as described by Martin *et al.* (1961). Storage of stocks of cells in ampoules at −70° made it possible to carry out all experiments on cells five generations removed from an original stock (Martin *et al.* 1961).

Washing and preincubation of Krebs cells. On withdrawal from mice, the ascitic fluid was transferred to 50ml. centrifuge tubes each containing one-third of their volume of the appropriate washing medium and centrifuged at 60*g* for 8min. at the temperature indicated in the individual experiments. The supernatant fluid was discarded and the sedimented cells were washed twice by centrifugation as above. Incubations were carried out at 2×10^7 cells/ml., usually under semi-anaerobic conditions, i.e. large volumes of cell suspensions in relatively small (2–5 times the volume of cell suspension) stoppered flasks incubated with only gentle shaking. The washing and incubation procedures were done with sterile solutions and glassware.

Preparation of ribosome and cell-sap fractions. After cells had been harvested, washed and incubated as required by the individual experiments, they were chilled on ice and, if necessary, washed rapidly twice with ice-cold TBS to remove phosphate (a potential inhibitor in the cell-free system). The cells were then centrifuged at 2300*g* for 3min. at 4°. In some instances the packed cells were stored at −70° before homogenization. If so, freezing and thawing were both done as rapidly as possible. The packed cells were resuspended at 0° in 3 times the packed-cell volume of 6mM-magnesium acetate. After standing at 0° for 2min., the cells were disrupted by about 30 strokes of a tight-fitting all-glass Dounce homogenizer. Immediately after homogenization, a volume of solution A equivalent to three-fifths the packed-cell volume was added to the homogenate, which was then centrifuged twice at 20000*g* for 10min. at 0°, the fat layer and sediment being discarded each time. The supernatant fluid was centrifuged at 105000*g* for 90min. at 0°. The fat layer was discarded and the remainder of the supernatant fluid pressure-dialysed (at 8lb./in.^{2}) overnight at 4° against a large volume of solution C. After centrifuging at 12000*g* for 10min. at 0°, this dialysed supernatant solution (termed 'cell sap') was stored at −18° or −70°. It is stable (with respect to activity in the cell-free system) for about a week at −18° or several weeks at −70°, but its activity is lost if it is refrozen.

Pellets from the 105000*g* centrifugation were resuspended in a small volume of ice-cold solution B by gentle hand homogenization in a small Potter homogenizer. To this microsome suspension was added 5% (w/v) sodium deoxycholate to give a final concentration of 0·5%. After standing on ice for 2min., the mixture was diluted with 30vol. of ice-cold solution B and the diluted suspension was centrifuged at 105000*g* for 90min. at 0°. The supernatant solution from this step was discarded and the ribosome pellets were rinsed, suspended in ice-cold 0·5M-sucrose and centrifuged at 12000*g* for 10min. to remove 'insoluble' material. The resultant supernatant ribosome suspension could be stored at −18° for several weeks. Once thawed, ribosomes were not refrozen and reused.

Amino acid-incorporation assay. The complete assay system used had a final volume of 1·0ml. and contained the following (final concentrations): GTP (0·25mM); ATP (1·0mM); phosphoenolpyruvate (5·0mM); pyruvate kinase (40μg./ml.); magnesium acetate (5·0mM); β-mercaptoethanol (20·0mM); tris–acetate buffer, pH7·7–7·8 (100mM); L-[^{14}C]phenylalanine of specific activity 9mc/m-mole (0·135μc); the 'other 19' amino acids (each 0·05mM); ribosomes equivalent to 2·0mg. of ribosomal protein and a saturating amount of cell sap. The K^+ concentration in the assay was adjusted to 50mM with KCl. Incubations were normally for 40min. at 37°. At the end of the incubation period the reaction mixtures were chilled and 7·0ml. of 5% (w/v) trichloroacetic acid was added to each. After standing for at least 20min. they were centrifuged, the supernatant fluid was discarded, and RNA and lipid were extracted from the pellets as described by Simkin & Work (1957). The dried protein was dissolved in 98% formic acid (A.R. grade) to give a final concentration of approx. 6mg. of protein/ml. An amount equivalent to 1·5–2·0mg. of protein was pipetted on to a tared aluminium planchet pretreated with silicone adhesive (cold-cure silastomer; Midlands Silicones Ltd., London, S.W. 1) to prevent curling of the protein films formed on drying under vacuum. The dried samples were weighed, then counted in a thin end-window Geiger–Müller counter. After corrections to

zero thickness (Calvin, Heidelberger, Reid, Tolbert & Yankwich, 1949), for counter background and zero-time assay (ribosomes held at 0° for 40 min. in the presence of ^{14}C-labelled amino acid for 40 min.), the results were expressed as counts/min./2 mg. of ribosomal protein, unless otherwise indicated. Alternatively, in some of the later experiments, the assays were scaled down to a final volume of 0·25 ml. and the procedure for radioactivity estimation after incubation was as follows: 0·25 ml. of N-NaOH containing an excess of unlabelled amino acids was added to each assay tube. After standing for 60 min. at room temperature, the protein was precipitated with 0·5 ml. of 30% (w/v) trichloroacetic acid, filtered on to an Oxoid membrane (Oxo Ltd., London), washed twice with cold 5% trichloroacetic acid and once each with 5% trichloroacetic acid at 90° and with diethyl ether–ethanol (2:1, v/v). The dry membrane was glued to an aluminium planchet and counted in a Nuclear-Chicago low-background counter (counting efficiency 16%). The results were expressed as counts/min./mg. of ribosomal protein, unless otherwise stated.

Preparation of EMC-virus RNA from crystalline virus. Growth, purification and crystallization of EMC virus were as described by Faulkner, Martin, Sved, Valentine & Work (1961). Crystalline virus was stored in PBS at −70° until required. To a freshly thawed crystalline virus suspension (approx. 2 mg. of virus/ml.) were added 5% (w/v) deoxycholate and 20 mM-EDTA, pH 7·2, to give final concentrations of 0·5% and 1 mM respectively (Ellem & Colter, 1961). The suspension was equilibrated at 45°; an equal volume of freshly redistilled water-saturated phenol at 45° was added and the mixture allowed to stand with occasional mixing for 10 min. at 40–45°. After cooling to 0° the mixture was centrifuged at 2300*g* for 15 min. at 4° and the aqueous layer removed. The phenol layer was re-extracted with 0·5 vol. of ice-cold water. The combined aqueous layers were mixed with 2 vol. of ethanol containing 2% (w/v) of potassium acetate and after 1 hr. at 0° the mixture was centrifuged at 2300*g* for 10 min. at 0°. The RNA pellet was washed by very gentle resuspension in ethanol–140 mM-NaCl (2:1, v/v) in a loose-fitting Dounce homogenizer. After recentrifugation as above, the pelleted material was stored at −70°.

For use in the cell-free system, the RNA was thawed, dissolved in distilled water to give a final concentration of approx. 1 mg./ml. and used immediately. The concentration of the RNA was estimated spectrophotometrically, $E^{1cm.}_{260}$ for 1 μg. of RNA/ml. being taken as 0·025.

Preparation of Krebs-cell ribosomal RNA. Ribosomal RNA was prepared from Krebs-cell ribosomes by the guanidinium chloride method of Cox & Arnstein (1963) with the modification (R. A. Cox, personal communication) that the RNA, after precipitation, was twice redissolved in 6 M-guanidinium chloride at room temperature and then precipitated with 0·5 vol. of ethanol at −18°, before being dissolved in water.

Krebs-cell s-RNA. RNA was extracted from Krebs cells by the method of Allen, Glassman & Schweet (1960*a*,*b*) and the s-RNA separated from the bulk RNA according to the $(NH_4)_2SO_4$-fractionation procedure of Littauer & Eisenberg (1959).

Estimation of ATP in Krebs cells. Cells were extracted with N-$HClO_4$ and the neutralized supernatant solutions were assayed for ATP by the hexokinase method (Kornberg, 1955).

RESULTS

Characteristics of the Krebs-cell ribosome–cell sap systems. The characteristics of the ribosome–cell sap systems of the type used throughout these studies are shown in Fig. 1 and Tables 1 and 2. In these systems amino acid incorporation ceases after approx. 30 min. at 37° (Fig. 1). It shows a linear dependence on ribosome concentration in the presence of saturating amounts of cell sap. For a given concentration of ribosomes, however, incorporation is dependent on the cell sap concentration up to that saturating level (Table 1). Results of this type were obtained whether the total incorporation observed was high or low. The systems show typical pH and Mg^{2+} concentration optima (Table 2) and a dependence on ATP and an ATP-generating system. Insensitivity to deoxyribonuclease treatment indicates the absence of any requirement for continuing DNA-mediated m-RNA synthesis. No stimulation of incorporation was observed on the addition of Krebs-cell s-RNA to any of these systems whether assayed in the presence or absence of EMC-virus RNA. It should be noted that throughout this work total incorporation rather than rate of incorporation was measured. Changes in activity therefore refer to changes in total incorporation, although, of course, the rate of incorporation may also be changing.

Effect of storage of Krebs cells at −70°. In some of the experiments described below, it proved convenient to store cells overnight at −70° before isolation of the ribosome and cell-sap fractions. The results of a number of control experiments

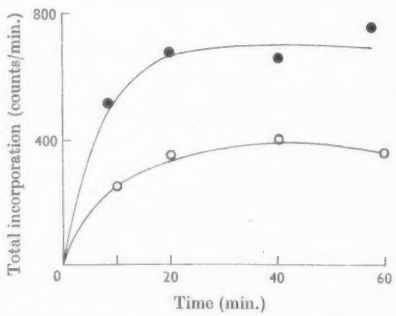

Fig. 1. Time-course of amino acid incorporation. Assays were as described in the Materials and Methods section, 2 mg. of ribosomal protein and 3 mg. of cell-sap protein being used in each assay. Systems were from: ●, cells washed at room temperature in TBS, then incubated for 4·25 hr. at 37° in Earle's medium; ○, cells washed at room temperature in TBS.

Table 1. *Characteristics of the Krebs-cell ribosome–cell sap system*

The complete system contained ribosomes equivalent to 2mg. of ribosomal protein and 3mg. of cell-sap protein. DL-[^{14}C]Leucine (7·2mc/m-mole) or L-[^{14}C]valine (6·5mc/m-mole) was substituted for L-[^{14}C]phenylalanine where indicated. Results are from duplicate experiments and are corrected relative to a value of 760counts/min. for the complete system containing phenylalanine (0·135μc). The composition of the complete system is given in the Materials and Methods section.

Assay system	Total incorporation (counts/min./assay)
Complete system	760
Complete system minus cell sap	80
Complete system with 1mg. of cell-sap protein	550
Complete system with 2mg. of cell-sap protein	700
Complete system with 4mg. of cell-sap protein	700
Complete system minus ribosomes	20
Complete system with 1mg. of ribosomal protein	360
Complete system with 1·5mg. of ribosomal protein	600
Complete system with 2·5mg. of ribosomal protein	925
Complete system assayed with DL-[^{14}C]-leucine	1340
Complete system assayed with L-[^{14}C]-valine	840
Complete system minus 'other 19' amino acids	350
Complete system minus ATP and ATP-generating system	50
Complete system plus deoxyribonuclease	760
Complete system plus Krebs-cell s-RNA (50μg.)	740
Complete system plus Krebs-cell s-RNA (200μg.)	710

with cells preincubated under a variety of conditions showed that storage of cells in this way did not significantly alter the activities of the ribosome–cell sap systems subsequently isolated from them.

Variation in the amino acid-incorporation activity of ribosome–cell sap systems from differently pretreated cells. Ribosome–cell sap systems prepared from cells that, on being harvested from mice, were washed rapidly in TBS or PBS at 0° (termed 'cold-washed TBS or PBS cells') supported high levels of incorporation (Table 3). Those prepared from cells washed at room temperature (18–22°) ('warm-washed TBS or PBS cells') were one-third to one-half as active. Further incubation of warm-washed TBS cells in TBS or PBS at 37° for 30min. ('warm-

Table 2. *Effect of* pH *and* Mg^{2+} *concentration on amino acid incorporation in the ribosome–cell sap systems*

Complete systems containing ribosomes equivalent to 2mg. of ribosomal protein and 5mg. of cell-sap protein were used throughout. Assays carried out between pH7·4 and 8·3 were 100mM with respect to tris–acetate buffer. Those performed between pH6·6 and 7·4 were 20mM with respect to tris–maleate–KOH buffer (the quantity of K^+ added in the form of KCl to each assay was adjusted for the K^+ concentration of the buffer solution). The composition of the complete system is given in the Materials and Methods section.

Concn. of Mg^{2+} (mM)	pH	Total incorporation (counts/min./2mg. of ribosomal protein)
2	7·8	320
5	7·8	760
7·5	7·8	695
10	7·8	640
12	7·8	480
5	6·6	550
5	7·0	750
5	7·4 (tris–maleate buffer)	820
5	7·4 (tris–acetate buffer)	900
5	7·6	930
5	7·8	960
5	8·0	650
5	8·3	200

Table 3. *Effect of preincubation of cells on the ribosome and cell-sap fractions*

Each system contained an amount of ribosomes equivalent to 2mg. of ribosomal protein and a saturating amount of cell sap (5mg. of cell-sap protein/assay). The composition of the complete system is given in the Materials and Methods section. Details of washing procedure and preincubation of cells are given in the Results section.

Source of ribosomes	Source of cell sap	Total incorporation (counts/min./2mg. of ribosomal protein)
Cold-washed TBS cells	Cold-washed TBS cells	860
Cold-washed TBS cells	Warm-washed preincubated TBS cells	870
Cold-washed TBS cells	Warm-washed TBS cells	878
Warm-washed TBS cells	Warm-washed TBS cells	358
Warm-washed preincubated TBS cells	Warm-washed TBS cells	40

washed preincubated TBS or PBS cells') yielded systems of very low activity. The results of an experiment in which each of the ribosome preparations from these differently pretreated cells was assayed with saturating amounts of a single cell-sap fraction and saturating amounts of each of the cell-sap fractions were assayed with a single ribosome fraction are presented in Table 3. Clearly, the lower activity of the systems from warm-washed and warm-washed preincubated cells resulted from a lowered activity of the ribosome fractions. This decrease in ribosomal activity observed on preincubation of cells in a simple buffered salts medium is referred to below as the 'depression' phenomenon.

The activity of ribosomes from cold-washed and warm-washed cells is also time-dependent, e.g. ribosomes from cells held for 3hr. at 0° in TBS displayed about one-half the activity of ribosomes from cells harvested and washed rapidly in the cold. To obtain reproducible results it was therefore essential to keep the time taken for harvesting and washing cells reasonably constant.

The recovery phenomenon. Warm-washed and warm-washed preincubated TBS cells, which would yield ribosomes with low activity, were incubated for various times at 37° in Earle's medium, and the ribosomes isolated and assayed with a single cell-sap fraction. Within 20min. incubation in Earle's medium, the cells yielded ribosomes that were again capable of supporting a high level of incorporation in the cell-free system (Fig. 2). To establish the factors necessary for this 'recovery' to occur, cells washed at room temperature in TBS were incubated at 37° in a variety of modified Earle's media and the ribosomes from them were assayed as indicated in Table 4. Clearly, neither Ca^{2+} and Mg^{2+} nor sodium hydrogen carbonate is essential for recovery, but glucose is. The results of Expt. 2 in Table 4 also show that the depression phenomenon occurs on incubation of cells in Earle's medium minus glucose, as well as in the less complex TBS medium.

Basis of the depression and recovery phenomena. Having defined the conditions necessary for the

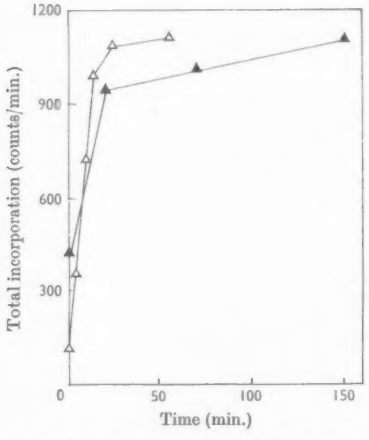

Fig. 2. Time-course of 'recovery' phenomenon. Warm-washed TBS cells (▲) and warm-washed preincubated TBS cells (△) were incubated for the times indicated at 37° in Earle's medium. Ribosomes were prepared and assayed as described in the Materials and Methods section with cell sap from zero-time cells.

Table 4. *Requirements for the 'recovery' phenomenon*

Ribosomes equivalent to 2mg. of ribosomal protein were used in each assay. All assays in Expt. 1 were carried out with cell sap from cells pretreated for 20min. at 37° in pseudo-Earle's medium, and in Expt. 2 with cell sap from cold-washed cells. The compositions of the incubation media are given in the Materials and Methods section.

Pretreatment of cells from which ribosomes were isolated	Total incorporation (counts/min./2mg. of ribosomal protein) Assayed with 3mg. of cell-sap protein	Assayed with 5mg. of cell-sap protein
Expt. 1. Cells were washed at room temperature in TBS then		
(*a*) incubated for 20min. at 37° in Earle's medium	960	1080
(*b*) incubated for 20min. at 37° in pseudo-Earle's medium	1060	1200
(*c*) incubated for 20min. at 37° in pseudo-Earle's medium minus Ca^{2+} and Mg^{2+}	880	1100
(*d*) incubated for 20min. at 37° in pseudo-Earle's medium minus $NaHCO_3$	960	1050
(*e*) incubated for 20min. at 0° in Earle's medium	500	500
Expt. 2 Cells were washed at room temperature in TBS then		
(*a*) incubated for 20min. at 37° in Earle's medium	700	750
(*b*) held at 0° for 20min. in Earle's medium	550	580
(*c*) incubated for 20min. at 37° in Earle's medium minus glucose	300	295

Table 5. *Effect of actinomycin D on the 'depression' and 'recovery' phenomena*

Cells were harvested, washed in PBS at 0°, suspended in Earle's medium at 2×10^7 cells/ml. and divided into two batches. Actinomycin D (5 μg./ml.) was added to one batch and both were left overnight at 4°. They were then incubated for 1 hr. at 37° and washed twice in PBS and once in TBS at room temperature. A third of each batch was stored as packed cells at −70° (warm-washed TBS cells ± actinomycin D). Another third of each batch was incubated at 2×10^7 cells/ml. in TBS ± actinomycin D for 40 min. at 37°. The final third was resuspended in Earle's medium ± actinomycin D at 2×10^7 cells/ml. and incubated for 40 min. at 37°. Ribosomes from all six batches were prepared and assayed ± saturating amounts of EMC-virus RNA with cell sap from non-actinomycin D-treated cells. The alternative scaled-down amino acid-incorporation procedure was used (see the Materials and Methods section), with DL-[^{14}C]leucine (12 mc/m-mole).

Source of ribosomes	Total incorporation (counts/min./mg. of ribosomal protein)		Percentage of warm-washed TBS-cell ribosomes (no RNA)
	No RNA	+ EMC-virus RNA	
Warm-washed TBS cells	618	1678	100
Warm-washed preincubated TBS cells	211	1185	34·1
Warm-washed TBS cells incubated in Earle's medium	1396	2849	225·9
Warm-washed TBS cells (+ actinomycin D)	376	1724	100
Warm-washed preincubated TBS cells (+ actinomycin D)	132	805	35·1
Warm-washed TBS cells incubated in Earle's medium (+ actinomycin D)	1103	2709	293·4

production of ribosomes capable of supporting different levels of incorporations, an attempt was made to identify the factors or factor responsible for these differences.

In the absence of any evidence for gross structural changes in the ribosomes or ribosomal RNA it seemed possible that the differing activities of the ribosome preparations might reflect differences in their m-RNA content. To investigate this possibility the effect of actinomycin D, a known inhibitor of DNA-directed RNA synthesis (Goldberg, Rabinowitz & Reich, 1962; Hurwitz, Furth, Malamy & Alexander, 1962), on the recovery phenomenon was studied and the m-RNA contents of the high- and low-activity ribosomes were compared.

The results of the actinomycin D experiment are shown in Table 5. The presence of the drug during preincubation of the cells, whether in the presence or absence of glucose, lowers the activity of the isolated ribosome fractions compared with controls. However, the general pattern of depression and recovery was unaffected. Since actinomycin D in the concentrations used here is known to inhibit 97–98% of all RNA synthesis in Krebs cells (Horton, Liu, Martin & Work, 1966), it seems unlikely that depression and recovery depend on the breakdown and synthesis of RNA.

Since most of the experimental criteria for m-RNA are rather arbitrary, it is difficult to measure m-RNA directly. However, the ability of an RNA preparation to stimulate amino acid incorporation in the cell-free system may be taken as some measure of its messenger content.

Table 6. *Stimulation of amino acid incorporation in the reticulocyte ribosome–pH 5 enzyme system by Krebs-cell 'ribosomal' RNA*

The rabbit-reticulocyte light-ribosome assay system (Arnstein *et al.* 1964) was used with 0·25 mg. of reticulocyte ribosomes and 1·0 mμmole of DL-[^{14}C]leucine (160 mc/m-mole) per assay. The composition of the assay medium is given in the Materials and Methods section.

RNA added	Total incorporation (counts/min.)
None	818
11 μg. of reticulocyte ribosomal RNA	1172
22 μg. of reticulocyte ribosomal RNA	1500
12 μg. of RNA from ribosomes of cold-washed TBS cells	1038
24 μg. of RNA from ribosomes of cold-washed TBS cells	1201
12·25 μg. of RNA from ribosomes of warm-washed preincubated TBS cells	1193
24·5 μg. of RNA from ribosomes of warm-washed preincubated TBS cells	1303

RNA was therefore isolated from ribosomes of warm-washed preincubated and cold-washed cells by the guanidinium chloride method of Cox & Arnstein (1963). When analysed in the analytical ultracentrifuge the two preparations gave identical patterns with only the two normal ribosomal components visible. There was no evidence for any degradation of the RNA. The ability of these preparations to stimulate amino acid incorporation was compared in a reticulocyte ribosome–pH 5

enzyme system prepared by the standard method of Arnstein, Cox & Hunt (1964). Both preparations produced appreciable stimulations compared with reticulocyte ribosomal RNA (Table 6). Assuming these stimulations to be a true measure of m-RNA content, it would appear that there is little difference between the ribosomes of warm-washed preincubated and cold-washed TBS cells in this respect.

Further circumstantial evidence against any variation in the m-RNA content of these different ribosome preparations was provided by the results of experiments to determine their polysome content. Preliminary studies involving sucrose-density-gradient analysis of ribosomes from cold-washed and warm-washed preincubated TBS cells have not, within the limitations of the technique, revealed differences in polysome content.

Variation in the ATP content of cells during depression and recovery. Laws & Stickland (1963) found that washing Ehrlich mouse ascites-tumour cells profoundly altered the ability of these cells to synthesize ATP. The ATP content of the differently preincubated Krebs cells was therefore measured. The results presented in Table 7 show that incubation of warm-washed TBS cells at 37° in the absence of glucose produces a rapid fall in the intracellular ATP content. On the addition of glucose and further incubation, the ATP content of such cells 'recovers' rapidly to the control level. Samples of the cells incubated as shown in Table 7 were used for ribosome preparation and the activity of the ribosomes was assayed with a single cell-sap fraction. The pattern of 'depression' and 'recovery' of ribosomal activity, as shown in Table 7, follows the pattern of change in ATP content in Table 7. This, however, does not prove a direct relationship between the intracellular ATP content and the ribosomal activity, but merely indicates that environmental conditions producing changes in the intracellular ATP content also produce changes in the activity of the ribosomes, in the same direction.

Stimulation of amino acid incorporation in the cell-free system by EMC-virus RNA. It might reasonably be expected that ribosomes deficient in m-RNA would respond well to the addition of exogenous messenger. It was therefore decided to compare the responses of the different ribosome preparations described above to EMC-virus RNA and polyU.

The EMC-virus RNA used throughout these studies was isolated from crystalline virus (Faulkner *et al.* 1961) by a modified phenol extraction technique. It consisted of approx. 50% of non-degraded 35s material and was highly infective when assayed in mice (10^7 LD_{50} units/mg. of RNA) or by a plaque assay technique (10^8 plaque-forming units/mg. of RNA).

Both EMC-virus RNA and polyU stimulate amino acid incorporation in the high- and low-activity ribosome–cell sap systems described above (Table 5 and Fig. 3). Similar stimulations were not observed with total Krebs-cell RNA, Krebs-cell s-RNA or degraded (<4s) EMC-virus RNA. In accord with results obtained with other cell-free systems and ribonucleic acids (Arnstein, Cox & Hunt, 1962; Davies, Gilbert & Gorini, 1964; Szer & Ochoa, 1964), the stimulation by EMC-virus RNA and polyU both show sharp and distinct Mg^{2+}-

Table 7. *Variation in ATP content and the activity of the ribosomes in differently preincubated Krebs cells*

Warm-washed TBS cells were incubated as shown below at a concentration of 2×10^7 cells/ml. The glucose concentration used is that present in Earle's medium. Samples (0·5 ml.) were taken at the end of the incubations for assay of the ATP content as described in the Materials and Methods section. The bulk of the cells were used to prepare ribosomes that were then assayed with DL-[1-^{14}C]leucine (12 mc/m-mole) in the scaled-down amino acid-incorporation assay (see the Materials and Methods section). Each tube contained 500 μg. of ribosomal protein and 1·14 mg. of cell-sap protein.

Treatment of cells	ATP content (μg./10^7 cells)	Total incorporation (counts/min./mg. of ribosomal protein)
In TBS at 0° for 60 min.	30·2	590
In TBS at 37° for 20 min., then at 0° for 40 min.	8·9	193
In TBS at 37° for 40 min., then at 0° for 20 min.	17·0	196
In TBS + 2 mg. of glucose/ml. at 37° for 40 min., then at 0° for 20 min.	45·4	1524
In TBS at 37° for 20 min., then in TBS + 2 mg. of glucose/ml. for 20 min. at 37°, followed by 20 min. at 0°	49·8	2944
In TBS at 37° for 40 min., then in TBS + 2 mg. of glucose/ml. for 20 min. at 37°	45·6	1564

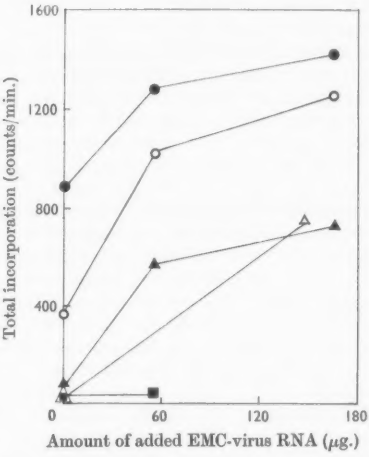

Fig. 3. Comparison of the response of ribosomes from differently preincubated cells to EMC-virus RNA. Each system contained ribosomes equivalent to 2mg. of ribosomal protein and a saturating amount of cell sap (5mg. of cell-sap protein/assay). EMC-virus RNA in the amounts shown was added to the following cell-free systems: ●, cold-washed TBS-cell system; ○, warm-washed TBS-cell system; ▲, warm-washed preincubated TBS/cell system; △, system of warm-washed preincubated TBS-cell ribosomes and cold-washed TBS-cell cell sap; ■, cold-washed TBS-cell cell sap (i.e. as for ● but minus ribosomes).

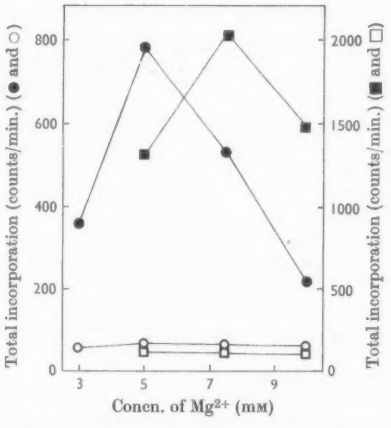

Fig. 4. Mg^{2+} concentration dependence of the response of cell-free systems to EMC-virus RNA and to polyU. Systems contained warm-washed preincubated TBS-cell ribosomes equivalent to 2mg. of ribosomal protein and saturating amounts of cold-washed TBS-cell cell sap (5mg. of cell-sap protein/assay): □, no polyU; ■, +20μg. of polyU; ○, no EMC-virus RNA; ●, +150μg. of EMC-virus RNA.

concentration optima (Fig. 4). With a depressed ribosome system, it was shown that approx. 60% of the total incorporation after stimulation by EMC-virus RNA was into supernatant protein and that at least 60% of this sedimented in a sucrose density gradient with a velocity of 2–8s with a broad peak at 2·6s (I. M. Kerr, M. G. Hamilton & T. S. Work, unpublished results). Thus it appears that a significant proportion of the amino acid incorporated in response to EMC-virus RNA is incorporated into high-molecular-weight polypeptide.

As shown in Fig. 3 and Table 5, the absolute stimulation of amino acid incorporation observed in response to saturating amounts of EMC-virus RNA does not differ widely for ribosomes of very different activities from cold-washed, warm-washed and warm-washed preincubated TBS cells. Moreover, there was no evidence to indicate that any factor other than the ribosome concentration was limiting in the final level of incorporation observed with the high-activity ribosomes from cold-washed cells in the presence of saturating amounts of EMC-virus RNA. Similar results have been obtained with polyU in place of EMC-virus RNA. The small amount of RNA required to saturate the system, and the fact that the levels of incorporation achieved with a high-activity system saturated with EMC-virus RNA always considerably exceed that observed with a similarly saturated low-activity system, suggests that only a fraction of the ribosomes is capable of responding to the added RNA. Further, this fraction appears to be relatively, if not completely, unaffected by the depression and recovery phenomena (Fig. 3 and Table 5).

Further tentative evidence in favour of a catalytic turnover of EMC-virus RNA through a limited number of ribosomes is provided by the kinetics of the amino acid incorporation after stimulation by EMC-virus RNA (Fig. 5). Curve *E* in Fig. 5, which represents the time-course of incorporation stimulated by EMC-virus RNA, shows that there is virtually no stimulation at 5min. A similar time lag has been reported for the tobacco-mosaic-virus-RNA–*Escherichia coli* cell-free system (Barondes & Nirenberg, 1962) and for polyU-stimulated incorporation in the rabbit-reticulocyte ribosomal system (Arlinghaus & Schweet, 1962). The reason for this lag is not known, but it may represent the time required for the attachment of EMC-virus RNA to the ribosomes and the synthesis of peptide chains that are sufficiently long to be precipitated in the assay procedure used. At 10min., but not at 40min., the system was saturated by 60μg. of EMC-virus RNA (curves *B* and *C* in Fig. 5), suggesting that EMC-virus RNA in excess of 60μg. added at zero time functions by maintaining incorporation over a longer period rather than by

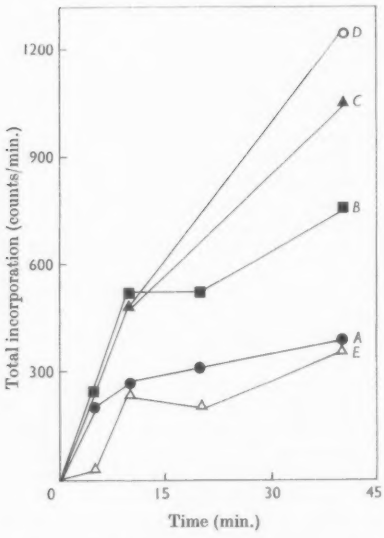

Fig. 5. Kinetics of stimulation by EMC-virus RNA. Each assay contained warm-washed TBS-cell ribosomes equivalent to 2 mg. of ribosomal protein and a saturating amount of cold-washed TBS-cell cell sap (5 mg. of cell-sap protein/assay): ●, no added RNA (curve *A*); ■, 60 μg. of EMC-virus RNA at zero time (curve *B*); ▲, 150 μg. of EMC-virus RNA at zero time (curve *C*); ○, 150 μg. of EMC-virus RNA at zero time + further 60 μg. at 10 min. (curve D); △, net stimulation due to 60 μg. of EMC-virus RNA (curve *E*).

increasing the initial rate. It seemed therefore that optimum stimulation by EMC-virus RNA might be achieved by successive additions throughout the incubation period. A comparison of curves *C* and *D* in Fig. 5 shows that the extent of incorporation can indeed be increased in this way.

The low-activity ribosomes produced by preincubation of the cells as described above gave a several-fold greater absolute response to EMC-virus RNA than did Krebs-cell ribosomes preincubated under the conditions of the amino acid-incorporation assay, as described by Nirenberg & Matthaei (1961).

DISCUSSION

Experiments with actinomycin D (Staehelin, Wettstein & Noll, 1963) have shown that the average half-life of m-RNA in mammalian cells is much greater than in bacteria (Levinthal, Keynan & Higa, 1962). In view of this it seemed likely that a mechanism might exist for controlling protein synthesis at some level besides that of gene transcription. Such a mechanism could operate at the ribosome level.

We have demonstrated that a cell-free ribosome–cell sap preparation from Krebs cells shows remarkable variability in its protein-synthesizing ability according to the previous history of the cells. This variability cannot be attributed entirely to a variation in the m-RNA content of the system. Recovery of ribosome activity when glucose-starved cells were reincubated in glucose was much too rapid to be attributed to messenger synthesis and in any case 'recovery' occurred in the presence of actinomycin D. The presence of extractable 'messenger' in an inactive ribosome preparation suggests that lack of messenger is not the explanation of the low activity of the ribosome–cell sap preparations from glucose-starved cells. Our colleague D. C. Dumonde (personal communication: Dumonde, Bitensky, Cunningham & Chayen, 1965) has found that there is increased lysosomal breakdown when Ehrlich cells are incubated in the absence of glucose and we cannot completely exclude the possibility that fragmented m-RNA is active when extracted and tested in a reticulocyte ribosome system, but we consider this explanation improbable. It seems more likely to us that the m-RNA–ribosome complex can exist in an active or in an inactive state and that in the inactive state the messenger remains in contact with the ribosomes but in a non-functional and 'protected' state. Recent work on reticulocytes and on sea-urchin eggs encourages us in this view. Thus Marks, Burka, Conconi, Perl & Rifkind (1965) have found that incubation of reticulocytes with sodium fluoride can convert rabbit-reticulocyte ribosomes into an inactive form but that cell recovery occurs promptly if fluoride is removed and glucose is added to the reticulocytes. In this system new messenger certainly cannot be formed as there is no nucleus. It is also relevant that Hultin (1964) showed the conversion of inactive into active ribosomes after fertilization of sea-urchin eggs. This conversion is energy-dependent but does not require messenger synthesis. In this respect the fertilized sea-urchin egg resembles the starved Krebs cell or the fluoride-treated reticulocyte in that it contains a population of ribosomes that can exist in an inactive or in an active form; in all three examples the conversion of the active form appears to be energy-dependent.

These results therefore strongly suggest that in the Krebs cell and in many other cells a mechanism exists for controlling the rate and extent of protein synthesis at the level of the ribosome independently of other control mechanisms that operate at the level of gene transcription. The response of 'inactive' ribosome preparations to added EMC-virus RNA shows that only a portion of the ribosomes

undergo this active–inactive transformation and that the proportion of EMC-virus-RNA-responsive ribosomes (presumably monomers) does not alter during cell starvation.

The above experiments do not yet provide an adequate basis for the understanding of the depression and recovery phenomena or of the factors controlling the response of ribosomal preparations of different intrinsic activities to EMC-virus RNA. However, from a purely practical point of view, these results are of considerable interest, for any variation in the time or temperature of pretreatment of cells can appreciably alter the activity *in vitro* of ribosomes subsequently isolated from them. It is therefore essential that in a comparison of the activities of ribosomes from differently pretreated cells the procedures used in the preparation of the cells for ribosomal isolation should be rigidly controlled. These results indicate that the relative amino acid-incorporation activities of ribosome preparations, assayed in the cell-free system, do not necessarily reflect their m-RNA content or that of the cells from which they were isolated, although under certain conditions they may undoubtedly do so. The response of such systems to the addition of exogenous m-RNA in the form of EMC-virus RNA or polyU appears to bear no necessary correlation to their intrinsic activities.

We thank Dr A. J. Morris for assistance in developing the cell-free system. Mr J. Coote provided skilled technical assistance.

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The Effect of Castration on the Ribonucleic Acid Metabolism of an Experimental Prostatic Tumour

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1. The incorporation of [^{14}C]phenylalanine into the protein of microsomes obtained from an androgen-dependent transplantable prostatic tumour has been investigated. 2. The incorporation of [^{14}C]ATP into the RNA of the nuclei from the same tumour has also been examined. 3. The castration of the host animal depresses the incorporation of both labelled compounds on subsequent incubation *in vitro*. 4. The uptake of phenylalanine can be markedly stimulated by polyuridylic acid only in tumours obtained from castrated host animals. 5. The tumour RNA-polymerase activity appears to be dependent on the concentrations of androgens in the host animal.

The prostate gland is one of the best examples of a strongly androgen-dependent tissue. It has been established that androgens control the growth of the prostate, at least in part, by their influence on RNA synthesis in the gland. Evidence has been presented that male steroid hormones control the rate of messenger-RNA synthesis (Liao & Williams-Ashman, 1962) and that the response is mediated via the nuclear RNA polymerase (DNA-dependent ribonucleotide triphosphate–ribonucleic acid nucleotidyltransferase: EC 2.7.7.6) (Hancock, Zelis, Shaw & Williams-Ashman, 1962). Little is known about the biochemical response of prostatic tumours to androgens, although the arrest of such carcinomas by castration alone or with combined oestrogen therapy is a successful clinical treatment.

The evaluation of the metabolic processes by which androgens regulate the growth of prostatic tumours has been severely hindered by the shortage of suitable hormone-responsive transplantable tumours. Induction of prostatic carcinomas in experimental animals by chemical means produces tumours that do not generally show the marked hormone dependency of the parent tissue, e.g. the methylcholanthrene-induced tumours described by Dunning, Curtis & Segaloff (1946).

Experiments have been carried out on an androgen-dependent prostatic tumour to establish whether a partial explanation of the steroid action could be found at the level of messenger-RNA synthesis, as in the non-malignant tissue. Messenger-RNA synthesis has been measured indirectly (e.g. Liao & Williams-Ashman, 1962; Pedersen & Hultin, 1963) by using a protein-synthesizing system and following the incorporation of [^{14}C]-phenylalanine into peptide linkage. The response of the system to added polyU† can be used as an indication of the amount of natural messenger RNA present in the system. Alternatively, the synthesis of RNA (including messenger RNA) may be followed by measurement of the activity of RNA polymerase in the nuclear fraction of tissue homogenates (e.g. Weiss, 1960; Widnell & Tata, 1964). The preparation of uncontaminated nuclei from solid tumours presents considerable practical difficulties, unless special homogenization equipment is used, such as that designed by Emanuel & Chaikoff (1957). A combination of such methods used *in vitro* has been used in the present investigation.

MATERIALS AND METHODS

Chemicals. ATP, CTP, GTP and UTP were purchased from P–L Biochemicals Inc., Milwaukee, Wis., U.S.A. Phosphoenolpyruvate (sodium salt) and pyruvate kinase were products of C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Solutions of the phosphate-containing compounds were titrated to pH 7·8 with N-NaOH before use. PolyU was purchased from Koch–Light Laboratories Ltd., Colnbrook, Bucks., and L-amino acids were obtained from British Drug Houses Ltd., Poole, Dorset. L-[U-^{14}C]-Phenylalanine (282 mc/m-mole) and [^{14}C]AMP (8 mc/m-mole) were obtained from The Radiochemical Centre, Amersham, Bucks. The [^{14}C]AMP was converted enzymically into [^{14}C]ATP in 43% yield by the method of Ochoa, Burma, Kröger & Weill (1961). Puromycin was the product of Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Actinomycin D was a generous gift from Merck,

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† Abbreviation: polyU, polyuridylic acid.

Sharp and Dohme, Rahway, N.J., U.S.A. Hyamine 10-X (Hopkin and Williams Ltd., Chadwell Heath, Essex) was converted into the hydroxide form by the method of Eisenberg (1958).

Chemical analysis. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine serum albumin as standard. RNA was determined by the method of Mejbaum (1939), with highly polymerized yeast RNA as reference; DNA was assayed by the procedure of Burton (1956), with highly polymerized calf-thymus DNA as standard.

Prostatic tumour. The prostatic carcinoma used in these experiments was developed by the implantation of 7,12-dimethylbenz[*a*]anthracene into the ventral prostate glands of male Sprague–Dawley rats. Pellets were made by mixing the carcinogen with twice its weight of paraffin wax (m.p. approx. 50°) and heating until the wax just melted. The mixture was thoroughly agitated, poured into appropriate moulds and allowed to cool. Pellets (about 100mg.) were implanted surgically into the ventral prostate glands of a group of eight rats that were then maintained under normal laboratory conditions. After about 4 months three of the animals developed palpable growths in the region of the prostate gland. The animals were killed and the least necrotic parts of the tumours were surgically transplanted into the livers of male Sprague–Dawley rats (approx. 200g.). Tumours were thereafter transplanted into the spleens of rats. At 4–5 weeks after transplantation the tumours had reached a palpable size and the animals were castrated or sham-operated via the scrotal route under ether anaesthesia. Groups of 9–12 tumours (each weighing 1·2–3g.) were used 7–12 days after the operations. The tumours showed a marked androgen-dependency in the early transplantations, since castration resulted in a 40–45% decrease in the average weight of the tumours. After repeated passage through many transplantations, however, the tumours grew more slowly, became progressively necrotic and showed virtually no relationship to the androgenic status of the host. The experiments under discussion were all carried out on prostatic tumours from the early transplantations, where the hormone-dependency was consistently maintained.

Preparation of nuclear fractions. The outer areas of the tumours were carefully dissected free of the central necrotic tissue and cooled in ice. The pooled material was homogenized at 2° in 4vol. of 0·25M-sucrose by using a motor-driven Teflon pestle (Tri-R Instruments, Jamaica, N.Y., U.S.A.) at 1200 rev./min. for a total homogenization time of 45sec. The crude homogenate was centrifuged at 700*g* for 5min. and the resultant residue was rehomogenized for a further 30sec. in 15ml. of 0·25M-sucrose. The homogenate was layered over an equal volume of 0·33M-sucrose containing $MgCl_2$ (1mM) and centrifuged at 800*g* for 10min. The pellet of nuclei and cell debris was suspended in 20ml. of 0·25M-sucrose containing $MgCl_2$ (1mM) (medium A) and centrifuged at 150*g* for 10min. After the careful withdrawal of the supernatant, the purified nuclei were collected by further centrifuging at 700*g* for 5min. The collected nuclei were gently resuspended in 15ml. of medium A and the final purification procedure was repeated. The final nuclear preparation was stored in 2·0ml. of medium A.

Extracts of the nuclear preparations and of the whole tumour tissue were prepared by the method of Schneider (1945). From the DNA content of these extracts, 35% of the original tumour DNA was recovered in the final preparation. The low yield of purified nuclei is a reflection of incomplete homogenization of the tumour; without exception, a heavy sediment of undegraded cellular material and debris followed the first low-speed purification step.

Preparation of microsomes and pH5 *enzyme fractions.* These were prepared as described by Mainwaring & Williams (1965) for uterine tumour material. The washed microsomes were suspended in 2·5ml. of a medium containing sucrose (0·15M), $MgCl_2$ (10mM), KCl (25mM) and tris buffer (35mM) adjusted to pH7·8 with HCl. The pH5 enzyme fraction was purified by passage through Sephadex G-25, equilibrated with a medium containing tris (10mM), $MgCl_2$ (10mM), KCl (5mM) and β-mercaptoethanol (5mM), adjusted to pH7·8 with HCl. In certain preliminary experiments, whole cell sap from the 105000*g* supernatant was used after purification by gel filtration.

Assay of RNA polymerase in the tumour nuclei. The assay system contained 0·3μmole each of CTP, GTP and UTP, 0·03μmole of [^{14}C]ATP, 3μmoles of NaF, 2·5μmoles of β-mercaptoethanol, 60μmoles of tris–HCl buffer, pH8·1, 15μmoles of KCl and 0·20ml. of nuclear suspension in a total volume of 0·5ml. The final $MgCl_2$ concentration was adjusted to 5mM. After incubation in air for 15min. at 37°, the reactions were stopped by the addition of 3·0ml. of ice-cold 0·8N-$HClO_4$, together with 0·2ml. of yeast RNA solution (10mg./ml.). The precipitates were collected by centrifuging at 2° and washed twice with ice-cold 0·3N-$HClO_4$, and finally twice in cold ethanol–ether (3:1, v/v). The extraction of the labelled RNA from the precipitates was carried out as described by Mainwaring & Williams (1965). The final extracts were dissolved in 0·5ml. of M-Hyamine hydroxide and added to 10ml. of scintillation medium (Bray, 1960). Radioactivity was measured by liquid-scintillation spectrometry at a counting efficiency of 65% (determined with [^{14}C]AMP under identical conditions).

Incorporation of [^{14}C]*phenylalanine into peptides.* The system contained 2μmoles of ATP, 0·4μmole of GTP, 10μmoles of phosphoenolpyruvate, 50μg. of pyruvate kinase, 10mμmoles (0·1μc) of L-[^{14}C]phenylalanine, 10mμmoles of each of the remaining 19 naturally occurring L-amino acids, 0·3ml. of pH5 fraction (or whole cell sap) and 0·25ml. of microsomal suspension in a final volume of 1·0ml. The final $MgCl_2$ concentration was adjusted to 12mM. Incubations were carried out in air at 37° for 1hr. The collection, washing and counting of the radioactive protein precipitates were carried out as described by Mainwaring & Williams (1965).

RESULTS

Incorporation of [^{14}C]*phenylalanine into the protein of prostatic-tumour microsomes.* The incorporation system was based on the experiments summarized in Table 1, with whole cell sap or the pH5 fraction as the source of activating enzymes and transfer RNA. Certain similarities are apparent between the experimental results in the two cases: (*a*) an energy-generating system is essential for activity; (*b*) the systems are sensitive to puromycin (Yarmolinsky & de la Haba, 1959); (*c*) purification of the enzymes by gel filtration is necessary for maximum incorporation of labelled phenylalanine.

Table 1. *Requirements for the incorporation of* [^{14}C]*phenylalanine with microsomes and either whole cell sap or* pH 5 *fraction*

The microsomes, whole cell sap and pH 5 fraction were prepared as described in the Materials and Methods section from tumours carried in normal male rats. The standard incorporation system contained 2 μmoles of ATP, 0·4 μmole of GTP, 10 μmoles of phosphoenolpyruvate, 50 μg. of pyruvate kinase, 10 mμmoles (0·1 μc) of L-[^{14}C]phenylalanine, 10 mμmoles of each of the remaining naturally occurring L-amino acids, 0·3 ml. of either (*a*) whole cell sap (4·4 mg. of protein) or (*b*) pH 5 fraction (3·2 mg. of protein) and 0·25 ml. of microsomal suspension (240 μg. of microsomal RNA) in a total volume of 1·0 ml. The final $MgCl_2$ concentration was adjusted to 12 mM. Incubations were for 1 hr. at 37°. Results are expressed as counts/min./250 μg. of microsomal RNA.

System	[^{14}C]Phenylalanine incorporated	
	(*a*) Whole cell sap	(*b*) pH 5 fraction
Complete system	661	854
With untreated cell sap or pH 5 fraction	150	306
Without ATP, phosphoenolpyruvate, pyruvate kinase	70	101
Without GTP	679	681
Without cell sap or pH 5 fraction	31	41
With final $MgCl_2$ concn. 5 mM	—	436
With final $MgCl_2$ concn. 10 mM	—	649
With added puromycin (30 μg.)	55	62
With added polyU (50 μg.)	445	1104
Preincubation of polyU + cell sap or pH 5 fraction, then added to complete system	320	1019

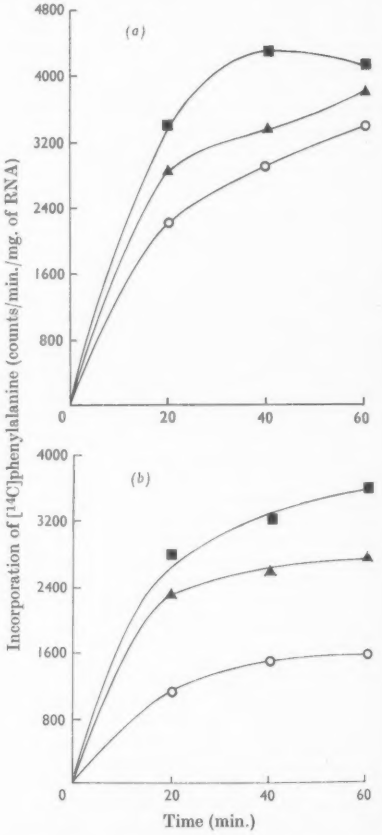

Fig. 1. Incorporation of [^{14}C]phenylalanine into protein (*a*) with microsomes and pH 5 fraction from sham-operated animals, (*b*) with microsomes from castrated animals and pH 5 enzymes from sham-operated animals. The constituents of the assay system are as described in the Materials and Methods section. Incorporation is expressed as counts/min./mg. of microsomal RNA. ○, No polyU; ▲, 25 μg. of polyU added; ■, 50 μg. of polyU added.

The low-molecular-weight inhibitor(s) has not been identified, but the presence of such material has been demonstrated in other tumour systems (Mainwaring & Williams, 1965). A marginal requirement for GTP was found only with the pH 5 enzyme fraction. In marked contrast, however, was the behaviour of the two systems to added polyU. With the whole cell sap as the source of enzymes, the activity of the system actually decreased in the presence of polyU; with pH 5 fractions, the synthetic messenger RNA caused a 20% increase in the incorporation of radioactivity. Degradation of synthetic polyribonucleotides by whole cell sap has been described by other workers, e.g. in *Escherichia coli* (Tissières & Watson, 1962) and Ehrlich ascites tumour (Pedersen & Hultin, 1963). It is uncertain whether such a degradation affords a satisfactory explanation of the above phenomenon, but preincubation of the polyU with whole cell sap before addition to the system resulted in a similar decrease in activity. This was not the case in a similar preincubation with the pH 5 fraction but the stimulation of incorporation was confirmed. If degradation of the polyU is the factor implicit in these findings, it suggests that the digestion products are strongly inhibitory to the system. The pH 5 fraction was used exclusively in the subsequent studies.

Comparison of the activities of tumour microsomes from castrated and sham-operated rats. Figs. 1(*a*) and 1(*b*) illustrate the incorporation of phenylalanine into the microsomal protein of tumours carried in control (sham-operated) and castrated rats respectively. The pH 5 enzyme fraction from

the control group was used in both cases. Since the yield of tumour microsomal RNA/g. of tumour was generally lower in the castrated group, the results have been corrected to give the radioactivity incorporated per unit amount of microsomal RNA.

The tumour microsomes from the castrated group were much more responsive to polyU than those derived from the controls. Without the synthetic messenger RNA, the system from castrated animals possessed low activity.

The direct addition of testosterone propionate to the system derived from the group of castrated animals did not enhance the activity of the protein-synthesizing system.

Synthesis of RNA in the tumour nuclear preparations. The conditions necessary for the synthesis of RNA in the isolated tumour nuclei are summarized in Table 2. With nuclei obtained from the control group (Table 2*a*) the incorporation of [^{14}C]ATP into RNA requires the presence of the remaining nitrogenous bases in the form of the 5′-triphosphates and the presence of both Mg^{2+} and β-mercaptoethanol. The incorporation of radioactivity was suppressed by the presence of ribonuclease to a background level. Actinomycin D, known to inhibit RNA polymerase by combination with the necessary DNA template (Cavalieri & Nemchin, 1964), was a potent inhibitor of the enzyme. In these respects, the requirements are similar to RNA-polymerase preparations obtained from other sources (Weiss, 1960; Gorski, 1964).

Table 2. *Incorporation of* [^{14}C]*ATP into RNA of tumour nuclei*

The nuclei (110–140 μg. of DNA) were obtained as described in the Materials and Methods section from sham-operated or castrated male rats. The complete system contained 0·3 μmole each of CTP, GTP and UTP, 0·03 μmole (0·05 μc) of [^{14}C]ATP, 3 μmoles of NaF, 2·5 μmoles of β-mercaptoethanol, 15 μmoles of KCl, 60 μmoles of tris–HCl buffer, pH 8·1, and 0·20 ml. of nuclear suspension in a total volume of 0·5 ml. The final $MgCl_2$ concentration was adjusted to 5 mM. Incubations were for 15 min. at 37°. Results are expressed as counts/min./125 μg. of DNA.

System	[^{14}C]ATP incorporated
(*a*) Control group	
Complete system	591
Without CTP, GTP, UTP	64
Without GTP	96
Without β-mercaptoethanol	263
With final $MgCl_2$ concn. 1 mM	146
With ribonuclease (50 μg.)	50
With actinomycin D (30 μg.)	40
(*b*) Castrated group	
Complete system	336
Without CTP, GTP, UTP	62
With actinomycin D (30 μg.)	31

Under standard conditions, the tumour nuclei from castrated animals have a decreased ability to synthesize RNA *in vitro* (Table 2*b*). These results suggest that castration of the tumour-bearing animals decreases the activity of the tumour RNA polymerase.

DISCUSSION

The experimental results are consistent with the view that the RNA synthesis in the prostatic tumour is dependent on physiological concentrations of androgens. This is indicated by the marked response of the tumour microsomal system to polyU after castration of the host animals. The control group showed only a small response to polyU, and Silverman, Liao & Williams-Ashman (1963) and Liao & Williams-Ashman (1962) have reported similar results with an incorporation system isolated from normal ventral rat prostate gland. The confirmatory experiments on the synthesis *in vitro* of RNA in the tumour nuclei suggest that a relationship exists between the synthesis of tumour RNA and the hormonal status of the animal. This is in agreement with the findings of Hancock *et al.* (1962) with aggregate RNA-polymerase preparations from normal prostatic nuclei.

There will almost certainly be changes in the concentrations of other hormones besides androgens after castration. The striking dependence of the normal prostate gland on physiological concentrations of androgens and the similar behaviour of the prostatic tumour does, however, suggest that the recorded effects were due to androgen depletion rather than to changes in the concentrations of other humoral agencies. Androgens may control the growth of the normal prostate gland by means of other metabolic parameters in addition to RNA synthesis. Control of the number of mitochondria (Edelman, Brendler, Zorgniotti & Edelman, 1963), the rate of fatty acid synthesis (Nyden & Williams-Ashman, 1953) and the permeability of membranes may all be implicated. Thus only a partial explanation of the response to androgens of the prostatic tumour would be expected at the level of RNA synthesis.

Even assuming this to be so, it leaves unsolved one of the biggest problems pertinent to the understanding of the biochemistry of the prostate gland and prostatic carcinomas. Adrion (1922), Francks (1954) and others have distinguished two distinct areas in the human prostate. They are distinctive in gross structure, tendency to neoplastic change and response to steroid hormones (both androgens and oestrogens). Thus a meaningful interpretation of the effects of androgens and other hormones on prostatic tissues must be related to characteristic

biochemical differences in the two morphological areas. This would involve more selective sampling of tissues than that employed in the present study.

The mechanisms by which the androgens regulate the activity of prostatic RNA polymerase has not been established so far. In a somewhat parallel set of circumstances in uterus, Talwar, Segal, Evans & Davidson (1964) have postulated that the response of uterine RNA polymerase to oestrogens involves de-repression of an enzyme. Such a mechanism may also be present in the prostate.

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The Secondary Structure of Ribosomal Ribonucleic Acid in Solution

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1. The u.v.-absorption spectrum of ribosomal RNA from rabbit reticulocytes was studied as a function of temperature at different pH values. The changes in the spectrum over the range 220–320 mμ were interpreted on the basis of the assumption that the effect of denaturation and ionization are additive. The results suggest that in neutral salt solutions the secondary structure of the ribosomal RNA samples studied is due to two species of helical segments stabilized principally, if not solely, by complementary base pairs but differing in nucleotide composition: each species appears to be heterogeneous in other respects in view of the breadth of the melting ranges. 2. The number of base pairs per helical segment was estimated to be small (between 4 and 17) on the basis of the relation between melting temperature and chain length previously established by Lipsett and others for model compounds. Small fragments (about 2s) obtained by alkaline hydrolysis appeared to form the same helical segments as the intact molecule in accord with the estimated size of these segments. 3. Specific nucleotide sequences appear necessary to account for the hysteresis observed on titrating ribosomal RNA with acid or alkali within the range pH 3·0–7·0 since this phenomenon was less pronounced for *Escherichia coli* transfer RNA and for RNA from turnip yellow-mosaic virus.

It appears that within the ribosome the RNA component forms, through intramolecular interactions, a stable helical secondary structure that is retained after dissociation of the protein moiety (for reviews see Arnstein, 1963; Petermann, 1964). A knowledge of the secondary structure of RNA in solution is relevant therefore to understanding the conformation of ribosomal RNA *in vivo*. Although the principal features of the secondary structure of RNA are known (for review see Spirin, 1963) further studies were initiated in an attempt to assess the contribution of specific nucleotide sequences to secondary structure, to identify the base pairs stabilizing helical segments and to estimate the size of helical segments.

The secondary structure of ribosomal RNA was characterized by studying the changes in the absorption spectrum brought about by increasing the temperature at different values of pH and electrolyte concentration. The results obtained on heating in neutral solutions were interpreted on the basis of the work of Felsenfeld & Sandeen (1962) and of Fresco, Klotz & Richards (1963). The changes in the spectrum of RNA noticed on heating at other pH values was interpreted on the basis of other studies (Cox, 1966; see also the Theoretical section). The results were correlated with an earlier approach (Cox, 1961; Cox and Arnstein, 1963) where the temperature was maintained constant and the pH was varied in order to yield the proportion of residues involved in secondary structure and the pH range over which cytosine, guanine and uracil residues were released from secondary structure.

The results of both types of experiment are consistent with the presence in ribosomal RNA from *Escherichia coli* and reticulocytes of at least two species of helical segments differing in nucleotide composition. The number of base pairs in the less thermally stable helical segments (approx. 50% of G + C) and in the more stable helical segments (approx. 85% of G + C) appears to be small (probably within the range 4–17). It is inferred from a comparison of the electrometric-titration properties of ribosomal RNA, TYMV* RNA and *E. coli* transfer RNA that specific nucleotide sequences give rise to the hysteresis noted on titration of ribosomal RNA over the range pH 3–7.

A short account of this work has been presented (Cox, 1965).

* Abbreviations: TYMV, turnip yellow-mosaic virus; A, G, U and C, adenine, guanine, uracil and cytosine residues respectively; oligoA etc., oligoadenylic acid etc.; polyA etc., polyadenylic acid etc., polyAU etc., copolymer of adenylic acid and uridylic acid etc.; poly(A + U) etc., complex of polyA and polyU etc.

THEORETICAL

The denaturation spectrum, i.e. the difference in $\epsilon_{(P)}$ (the molar extinction coefficient referred to 1 g.atom of P), over the range 220–300 mμ, found for the thermally induced transition from double-helical to amorphous forms of G--C and A--U pairs, namely:

$$\text{G--C} \rightleftharpoons \text{G--aq.} + \text{C--aq.} + \Delta\epsilon_{(P)}^{GC} \quad (1)$$

$$\text{A--U} \rightleftharpoons \text{A--aq.} + \text{U--aq.} + \Delta\epsilon_{(P)}^{AU} \quad (2)$$

(where -- indicates hydrogen bonds) are distinct (Felsenfeld & Sandeen, 1962; Fresco *et al.* 1963). The denaturation spectrum of G--C base pairs has maxima at 245 and 278 mμ, whereas the denaturation spectrum for A--U base pairs has a maximum at 262 mμ and both helical and amorphous forms have identical values of $\epsilon_{(P)}$ at 230 and 280 mμ.

The nucleotide composition of helical segments melting to an amorphous form over a particular temperature range has been estimated on the basis of the assumption that each base pair contributes independently to the denaturation spectrum, irrespective of its nearest neighbours (Felsenfeld & Sandeen, 1962; Fresco *et al.* 1963). This information may be derived from the ratio $\Delta E_{260}/\Delta E_{280}$ since:

$$\Delta E_{260}/\Delta E_{280} = (\delta f_{AU}\Delta\epsilon_{260}^{AU} + \delta f_{GC}\Delta\epsilon_{260}^{GC})/\delta f_{GC}/\Delta\epsilon_{280}^{GC} \quad (3)$$

where δf_{AU} and δf_{GC} are respectively the mole fractions of A--U and G--C base pairs denaturing over the particular temperature range studied. Eqn. (3) may be rearranged to:

$$\Delta E_{260}/\Delta E_{280} = (\delta f_{AU}/\delta f_{GC})(\Delta\epsilon_{260}^{AU}/\Delta\epsilon_{280}^{GC}) + \Delta\epsilon_{260}^{GC}/\Delta\epsilon_{280}^{GC} \quad (4)$$

The value of $\Delta\epsilon_{260}^{AU}$ was determined by Fresco *et al.* (1963) whereas $\Delta\epsilon_{260}^{GC}$ and $\Delta\epsilon_{280}^{GC}$ were obtained by placing the denaturation spectrum observed by Fresco *et al.* (1963) for polyGC on a molar basis (see Fig. 1*a*) by taking into account the fraction of cytosine residues involved in helical secondary structure (Cox, 1965, 1966). Hence by substitution in eqn. (4) and rearranging we have:

$$\delta f_{AU}/\delta f_{GC} = 0{\cdot}81(\Delta E_{260}/\Delta E_{280}) - 0{\cdot}46 \quad (5)$$

the relation utilized below.

When a polynucleotide is heated at constant pH in acidic or alkaline solutions its spectrum is affected both by changes in conformation and by changes in the degree of ionization of the base residues, partly owing to the dependence of the dissociation constant (pK) on the absolute temperature:

$$pK = A + B/T \quad (6)$$

where $A = 0{\cdot}65$ and $B = \Delta H/2{\cdot}303\boldsymbol{R}$, but principally owing to the effect of denaturation because the pK of a base residue may change by about 2 units as a result of the transition from the double-helical to the amorphous form. The forms of the denaturation spectra at pH values where the base residues ionize were deduced (see Fig. 1) on the basis of the assumption that the effects of ionization and denaturation are additive, and that the difference in the spectrum of the ionized and neutral forms of a base residue is the same as for the corresponding nucleotide. The limiting denaturation spectrum predicted for the transition from the double-helical form, in which the base residues are not ionized, to an amorphous form, in which the ionization of one species of base residue is complete, was calculated by summing the appropriate denaturation spectrum (for 1·0 g.atom of P) with the difference in the spectrum of ionized and neutral forms of the base residue (for 0·5 g.atom of P) concerned (see Fig. 1).

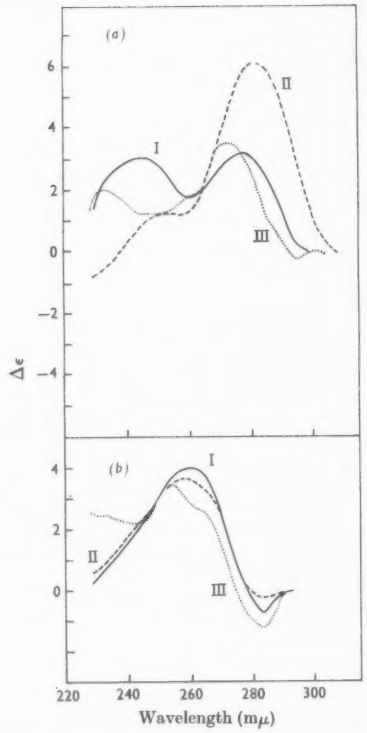

Fig. 1. Denaturation spectra of G--C and A--U base pairs in neutral, acidic and alkaline solutions. (*a*) Curve I, denaturation spectrum of G--C base pairs in neutral solutions [values of Fresco *et al.* (1963), X I.28; see Cox (1966)]; curve II, calculated denaturation spectrum of G--C base pairs at low pH values [curve I+0·5 ($\epsilon_{(P)\,pH1} - \epsilon_{(P)\,pH}$)CMP]; curve III, calculated spectrum for denaturation at high pH [curve I+0·5 ($\epsilon_{(P)\,pH12} - \epsilon_{(P)\,pH\,7}$)GMP]. (*b*) Curve I, denaturation spectrum of A--U base pairs at pH 7 (Fresco *et al.* 1963); curve II, calculated spectrum for denaturation at low pH [curve I+0·5 ($\epsilon_{(P)\,pH1} - \epsilon_{(P)\,pH\,7}$)AMP]; curve III, calculated denaturation spectrum at high pH [curve I+0·5 ($\epsilon_{(P)\,pH12} - \epsilon_{(P)\,pH\,7}$)].

Denaturation in acidic solutions. Thermal denaturation at a constant (acidic) pH affects A--U base pairs according to the equation:

$$\begin{array}{lcl} \text{A--U} & \text{Heat;} & \text{A--aq.} + \text{U--aq.} \\ \Downarrow\!\Uparrow\ pK \text{ approx } 3{\cdot}5 & \longrightarrow & \Downarrow\!\Uparrow\ pK \text{ approx. } 4{\cdot}5 \\ \text{AH}^+\text{--U} & \text{pH constant} & \text{AH}^+\text{--aq.} \end{array} \quad (7)$$

However, since the ionized and neutral forms of adenine have practically the same spectrum, the thermal denaturation spectrum remains effectively the same in acidic as in neutral solutions (Fig. 1*b*).

The analogous transition that G--C base pairs undergo on thermal denaturation in acidic (0·1 M-sodium phosphate) solutions is:

$$\begin{array}{ccc} \text{G--C} & \xrightarrow{\text{Heat;}} & \text{G--aq.}+\text{C--aq.} \\ \Downarrow pK \text{ approx. } 3{\cdot}1 & \text{pH constant} & \Uparrow pK = 4{\cdot}70 \\ \text{G--CH}^+ & & \text{CH}^+\text{--aq.} \end{array} \quad (8)$$

The left-hand side of eqn. (8) denotes that, in the double-helical form, cytosine residues ionize as a weak acid:

$$\text{CH}^+ \rightleftharpoons \text{C}+\text{H}^+ \quad (9)$$

where α is the degree of dissociation, $1-\alpha$ is the fraction of ionized cytosine residues and h is the H^+ concentration; and:

$$pK = \text{pH}+\log[(1-\alpha)/\alpha] \quad (10)$$

It has been shown (Dove & Davidson, 1962; Cox, 1966) that about 80% of cytosine residues may become protonated without disrupting the double-helical conformation. In the amorphous form the pK of cytosine residues is 4·70 at 25° (Cox, 1966).

In contrast with adenine residues there is a marked difference in the spectrum of the ionized and neutral forms of cytosine residues, particularly over the range 265–310 mμ. Hence the limiting denaturation spectrum for G--C base pairs (Fig. 1) is considerably enhanced at wavelengths longer than 265 mμ whereas $\Delta\epsilon_{260}$ is hardly affected. The extent to which the limiting spectrum is approached depends on the difference $\delta(1-\alpha)$ in the degree of ionization of cytosine residues as a result of the transition from helical to amorphous forms.

Thus for RNA:

$$\Delta E_{280}/\Delta E_{260} = [\delta f_{GC}\Delta\epsilon_{280}^{GC}+\delta(1-\alpha)\Delta\epsilon_{280}^{CH^+}]/(\delta f_{GC}\Delta\epsilon_{260}^{GC}+\delta f_{AU}\Delta\epsilon_{260}^{AU}) \quad (11)$$

Now:

$$\delta(1-\alpha) = [\partial(1-\alpha)/\partial f_{GC}]_T\,\delta f_{GC}+[\partial(1-\alpha)/\partial T]_{f_{GC}}\delta T \quad (12)$$

so that:

$$\Delta E_{280}/\Delta E_{260} = (\delta f_{GC}\Delta\epsilon_{280}^{GC}+\{[\partial(1-\alpha)/\partial f_{GC}]_T\,\delta_{GC}+[\partial(1-\alpha)/\partial T]_{f_{GC}}\delta T\}\Delta\epsilon_{280}^{CH^+})/(\delta f_{GC}\Delta\epsilon_{260}^{GC}+\delta f_{AU}\Delta\epsilon_{260}^{AU}) \quad (13)$$

where:

$$\Delta\epsilon_{280}^{CH^+} = \epsilon_{280}(\text{CMP, pH 1})-\epsilon_{280}(\text{CMP, pH 7})$$

and:

$$[\partial(1-\alpha)/\partial f_{GC}]_T = \{[K_H/(h+K_H)]-[K_a/(h+K_a)]\} \quad (14)$$

and:

$$[\partial(1-\alpha)/\partial T]_{f_{GC}} = -(B/T^2)e^{2{\cdot}3[A+(B/T)-\text{pH}]}(1+e)^{2{\cdot}3[A+(B/T)-\text{pH}]} \quad (15)$$

where $A = 0{\cdot}65$ and $B = 1{\cdot}22\times10^3$ for cytosine residues (see eqn. 6). Hence the opposing effects of denaturation and temperature on $1-\alpha$ may be precisely evaluated. For RNA in the amorphous form ($\delta f_{GC} = 0$) heating will lead to a decrease in $\Delta E_{280}/\Delta E_{260}$ according to eqn. (15). It is evident from eqn. (13) that maximum discrimination between denaturation of A--U and G--C base pairs is achieved when $[\partial(1-\alpha)/\partial f_{GC}]_T$ is maximum, i.e. when:

$$\text{pH} = \tfrac{1}{2}(pK_H+pK_a) \quad (16)$$

For cytosine residues at 25° the optimum is pH 3·9 but raising the temperature will lead to a decrease in the optimum pH. A value of about pH 3·9 is convenient for study since the helical secondary structure present at pH 7 persists on titration to about pH 4 at 25°.

Denaturation in alkaline solutions. Thermal denaturation at constant (alkaline) pH leads to the transitions:

$$\begin{array}{ccc} \text{A--U} & \xrightarrow{\text{Heat;}} & \text{A--aq.}+\text{U--aq.} \\ \Downarrow pK_H \gg 11{\cdot}4 & \text{pH constant} & \Uparrow pK = 9{\cdot}7 \\ \text{A--U}^-+\text{H}^+ & & \text{U}^-\text{--aq.}+\text{H}^+ \end{array} \quad (17)$$

and:

$$\begin{array}{ccc} \text{G--C} & \xrightarrow{\text{Heat;}} & \text{G--aq.}+\text{C--aq.} \\ \Downarrow pK_H \gg 11{\cdot}4 & \text{pH constant} & \Downarrow \\ \text{G}^-\text{--C}+\text{H}^+ & & \text{G}^-\text{--aq.} \end{array} \quad (18)$$

Values of pK_H are not known precisely because, in the double-helical form, ionization of the base residues is completely suppressed even at pH 11·4 (Cox, 1963*a*). On the basis of previous work a pK value of 9·70 (cf. Warner & Breslow, 1958) was assigned to both guanine and uracil residues. Thus it is possible, in principle, to bring about the transition from the double-helical form of a polynucleotide in which guanine and uracil residues are not ionized to an amorphous form in which they are completely ionized; the limiting denaturation spectra calculated for A--U and G--C base pairs are given in Fig. 1.

Ionization affects the spectra of both guanine and uracil residues so that the differences in the denaturation spectra are accentuated (Fig. 1), particularly in the region 260–280 mμ. Ionization of uracil residues leads to a decrease in extinction at wavelengths longer than 245 mμ and to an increase in $\epsilon_{(P)}$ at shorter wavelengths, whereas ionization of guanine residues leads to an increase in ϵ_{270}; ϵ_{230}, ϵ_{260} and ϵ_{278} are unchanged. Empirically it was found that $\Delta E_{270}/\Delta E_{260}$ is a convenient ratio that may be utilized to facilitate the discrimination of the melting ranges of helical segments differing in nucleotide composition.

For A--U base pairs:

$$\Delta E_{270}/\Delta E_{260} = (\delta f_{AU}\Delta\epsilon_{270}^{AU}+\delta\alpha_U\Delta\epsilon_{270}^{U^-})/(\delta f_{AU}\Delta\epsilon_{260}^{AU}+\delta\alpha_U\Delta\epsilon_{260}^{U^-}) \quad (19)$$

where:

$$\delta\alpha_U = (\partial f_U/\partial f_{AU})_T\,\delta f_{AU}+(\partial\alpha_U/\partial T)_{f_{AU}}\delta T \quad (20)$$

$\Delta E_{270}/\Delta E_{260}$ remained constant on titration of poly(A+U) with alkali (Cox, 1966).

For G--C base pairs:

$$\Delta E_{270}/\Delta E_{260} = (\delta f_{GC}\Delta\epsilon_{270}^{GC}+\delta\alpha_G\Delta\epsilon_{270}^{G^-})/(\delta f_{GC}\Delta\epsilon_{260}^{GC}+\delta\alpha_G\Delta\epsilon_{260}^{G^-}) \quad (21)$$

$\Delta\epsilon_{270}^{G^-}$ is positive and $\Delta\epsilon_{260}^{GC}$ is negligible so that in alkaline solutions $\Delta E_{270}/\Delta E_{260}$ is greater (1·75) than the value (1·3) obtained at pH 7 (Cox, 1966); and:

$$\delta\alpha_G = (\partial\alpha_G/\partial f_{GC})_T\,\delta f_{GC}+(\partial\alpha_G/\partial T)_{f_{GC}}\delta T \quad (22)$$

For RNA:

$$\Delta E_{270}/\Delta E_{260} = (\delta f_{AU}\Delta\epsilon_{270}^{AU}+\delta f_{GC}\Delta\epsilon_{270}^{GC}+\delta\alpha_U\Delta\epsilon_{270}^{U^-}+\delta\alpha_G\Delta\epsilon_{270})/(\delta f_{AU}\Delta\epsilon_{260}^{AU}+\delta f_{GC}\Delta\epsilon_{260}^{GC}+\delta\alpha_U\Delta\epsilon_{260}^{U^-}) \quad (23)$$

It is possible to show by substituting for different values of $\delta f_{AU}/\delta f_{GC}$, when $\alpha_G = \alpha_U = 1$, that $\Delta E_{270}/\Delta E_{260}$ increases as δf_{GC} increases.

For guanine and uracil residues the degree of ionization is the same as the degree of dissociation, so that raising the temperature will increase the degree of ionization of residues in amorphous segments and tend to increase the ratio $\Delta E_{270}/\Delta E_{260}$.

The changes in the spectrum, notably ΔE_{260}, ΔE_{270} and ΔE_{280}, on denaturation analysed above were studied principally for diagnostic purposes. The possibility of analysing the denaturation spectrum to yield δf_{AU}, δf_{GC}, $\delta\alpha_U$, $\delta\alpha_G$ and $\delta\alpha_C$ is examined elsewhere (Cox, 1966).

EXPERIMENTAL

Materials. RNA was isolated from rabbit reticulocytes by a modification (Cox, 1965) of the method of Cox & Arnstein (1963).

RNA was isolated from TYMV by extraction with aqueous phenol. The virus was first purified by differential centrifuging. TYMV (10 mg./ml.) in 0·05 M-KCl was shaken for 1 min. with an equal volume of cold phenol saturated with water. The aqueous layer was extracted a second time with phenol and the RNA was then precipitated with propan-2-ol. The precipitate was redissolved in water containing bentonite (2 mg./ml.) and stored at −20°. The sedimentation pattern of RNA in 0·05 M-KCl revealed that about 60% of the u.v.-absorbing species sedimented as one boundary, the sedimentation coefficient ($S_{20,w}$) being about 30 S. The remainder was characterized by $S_{20,w}$ 15–30 S.

Ribosomal RNA from *E. coli* was isolated by the method of Littauer & Eisenberg (1959).

The isolation of *E. coli* transfer RNA (which was given by E. M. Martin) was described by Martin, Yegian & Stent (1963).

Yeast RNA was isolated from whole cells by the method of Crestfield, Smith & Allen (1955). The sedimentation pattern showed that degradation to not less than 6 S fragments had occurred during isolation.

Crystallizable fragments (about 2·4 S; Spencer, Fuller, Wilkins & Brown, 1962) of yeast RNA were given by Dr G. L. Brown.

PolyAGUC, which was given by Dr Grunberg-Manago, was prepared by polymerizing ADP, GDP, UDP and CDP in the respective molar proportions 1:0·5:1:1 with polynucleotide phosphorylase. The product had $S_{20,w}$ about 2·5 S although the sedimenting boundary was broad.

Methods. A Unicam SP.700 spectrophotometer fitted with an electrically heated copper block that served as a cell holder (Cox, 1963*a*) was used for measuring extinctions in the range 220–320 mμ. The stoppered cuvette was weighed before and after heating to ensure that the weight loss due to evaporation was 1% or less. The extinction of the cooled solution was also measured, to verify that the changes in extinction were reversible to within ±1%, and in some cases the RNA sample was again heated to check the reproducibility of the dependence of extinction on temperature. Values of E were corrected for changes in concentration on heating due to the expansion of water.

The procedure used for electrometric titrations was as described by Cox & Arnstein (1963). The pH of the solution was measured by means of a micro-electrode covering the range pH 0–14 (Jena Glass combined electrode, 02779 type U; Jenaer Glasswork, Schott und Gen., Mainz, Germany) used in conjunction with an E.I.L. model 39A pH-meter (Electronic Instruments Ltd., Richmond, Surrey).

A Spinco model E ultracentrifuge fitted with u.v. optics was used for all analytical ultracentrifuging. The optical density of the photographic film was measured by means of a double-beam recording micro-densitometer (model E12 Mk IIIB; Joyce–Loebl and Co. Ltd., Newcastle upon Tyne).

RESULTS

Ribosomal RNA from reticulocytes. Ribosomal RNA from rabbit reticulocytes in 0·5 mM-, 10 mM- and 100 mM-sodium phosphate buffer, pH 7·6, was heated to temperatures in the range 25–75°. At each concentration of sodium phosphate the difference in the spectrum of the heated sample and that of a sample maintained at 23–25° was measured. The curves $(E_{(T)} - E_{(25)})$ for RNA in 0·5 mM-phosphate buffer are presented in Fig. 2(*a*). It is apparent that the difference curves have the same characteristic shape (curve I in Fig. 2*b*) in the range 25–50° (E_{280}/E_{260} 0·6), whereas the difference curves $(E_{(T)} - E_{(50)})$ in the range 50–75° are bimodal (curve II in Fig. 2*b*) with maxima at about 247 and 277 mμ and a minimum at 263 mμ (E_{280}/E_{260} 1·2).

The fraction of G+C in helical domains melting to an unfolded form over the range 25–50° was estimated as 50 ± 2% from the appropriate value of the ratio E_{280}/E_{260} (Fig. 3*a*) by using eqn. (5) compared with the value of 85 ± 5% of G+C deduced for domains melting above 50° (Fig. 3*b*).

The relative values of E_{260} in the range 25–75° given in Fig. 3(*c*) also reflect the biphasic nature of the transition from helical to unfolded forms. Thus the increase of 15% in E_{260} over the range 25–50° denotes the 'melting' of domains having about

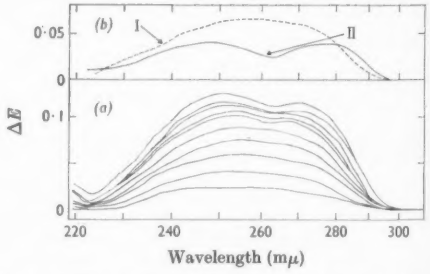

Fig. 2. Denaturation spectra $(E_{(T)} - E_{(25)})$ of reticulocyte ribosomal RNA in 0·5 mM-sodium phosphate buffer, pH 7·6. (*a*) T = 30, 35, 40, 45, 50, 55, 60, 65, 70 and 75° successively. (*b*) Curve I, $E_{(50)} - E_{(30)}$; curve II, $E_{(75)} - E_{(50)}$. At 25°, $E_{260} = 0{\cdot}655$.

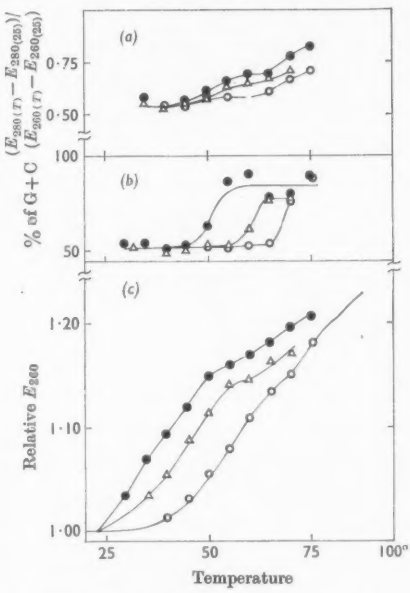

Fig. 3. Effect of electrolyte concentration on the thermal stability of the secondary structure of reticulocyte ribosomal RNA. (*a*) $(E_{280(T)}-E_{280(25)})/(E_{260(T)}-E_{260(25)})$. (*b*) % of G+C in helical segments denaturating at the temperature indicated, calculated by substituting values of $E_{280(T_2)}-E_{280(T_1)}$ and $E_{260(T_2)}-E_{260(T_1)}$ (where $T_2 > T_1$) in eqn. (5). (*c*) Dependence of $E_{260(T)}/E_{260(22)}$ on temperature. In each Figure ●, △ and ○ refer respectively to 0·5 mM-, 0·01 M- and 0·1 M-sodium phosphate buffer, pH 7·6.

50% of G+C (T_m 38°). The inflexion noticed over the range 50–55° marks the end of this transition. Above 55° the increase in E_{260} reflects the melting of domains richer in guanine and cytosine residues.

When the concentration of sodium phosphate was increased to 10 mM the difference curves observed were similar to those presented in Fig. 2 although higher temperatures had to be attained to bring about the same increase in extinction. Thus domains of about 50% of G+C 'melted' over the range 25–55° (T_m approx. 44°). Increasing the concentration of sodium phosphate to 100 mM further stabilized secondary structure so that still higher temperatures were required in order to 'melt' out helical domains, e.g. domains of about 57% of G+C 'melted' over the range 35–70°.

Further evidence for the presence of at least two distinct species of helical segments differing in $\delta f_{AU}/\delta f_{GC}$ was obtained by studying the changes in the spectrum of RNA on denaturation in acidic and alkaline solutions. No significant change in melting temperature was noticed over the range pH 4·5–7·0 but when the solution (0·1 M-sodium phosphate) was brought to pH 4·00 the changes in ΔE_{260} and ΔE_{280} took place over a slightly lower temperature range. Two distinct melting ranges were discerned. The lower-melting species was characterized by a $\Delta E_{280}/\Delta E_{260}$ value of about 0·80, which is higher than that (0·60) observed on denaturation at pH 7 but appreciably lower than value of 1·2 for $(E_{280(T)}-E_{280(66)})/(E_{260(T)}-E_{260(66)})$ observed over the range 66–86° (Fig. 4*c*).

When the pH was decreased to pH 3·60 the thermal stability of secondary structure was further diminished and it was easier to distinguish the melting ranges of the two species of helical segments as predicted in the Theoretical section. The dependence of both ΔE_{260} and ΔE_{280} on temperature were clearly biphasic; $\Delta_{280}/\Delta E_{260}$ was found to be 1·0 in the range 20–53° (compared with 0·6 at pH 7, and 1·8 at pH 4·00), whereas at higher temperatures $\Delta E_{280}/\Delta E_{260}$ was about 1·6 (cf. 1·2 in the range pH 4·0–7·02). Values of $(E_{280(T)}-E_{280(53)})/(E_{260(T)}-E_{260(53)})$ are given in Fig. 4(*b*). The denaturation spectrum in the range 25–50° was skewed towards longer wavelengths compared with that observed at pH 7·6 (curve I in Fig. 2*b*), the maximum being shifted from about 257 mμ at pH 7·6 to 273 mμ at pH 3·6 (curve I Fig. 5*b*) in accord with transitions (7) and (8). The denaturation spectrum in the range 50–85° more nearly approached the limiting spectrum for the denaturation of G--C base pairs in acidic solutions (eqn. 8); the maximum observed at 245 mμ at pH 7 was no longer apparent but the maximum at about 280 mμ was more prominent (curve II in Fig. 5*b*).

Decreasing the pH to 3·08 led to a further decrease in the thermal stability of helical secondary structure (Fig. 4*a*). The less stable species had already 'melted' at 25° at this pH, although the species richer in G--G base pairs persisted. The slight decrease in $\Delta E_{280}/\Delta E_{260}$, i.e. $(E_{280(T)}-E_{280(32)})/(E_{260(T)}-E_{260(32)})$, relative to pH 3·60 is attributed to the decrease in the term $(\partial\alpha/\partial f_{GC})_T$ (see eqn. 12).

When the solution was brought to pH 9·80 the secondary structure decreased slightly in thermal stability. The dependence of ΔE_{260} and ΔE_{270} on temperature was found to be biphasic (Fig. 4*e*) and each phase was characterized by a particular denaturation spectrum (Fig. 6). Over the range 25–50° the difference spectrum shown by curve I in Fig. 6(*b*) was observed; the maximum was found at 266 mμ and $\Delta E_{270}/\Delta E_{260}$ was about the same as at pH 7.

Curve II in Fig. 6(*b*) was typical of denaturation at higher temperatures; only one maximum, at 272 mμ, was noted, in contrast with maxima at 245 and 280 mμ at pH 7. Thus both species of

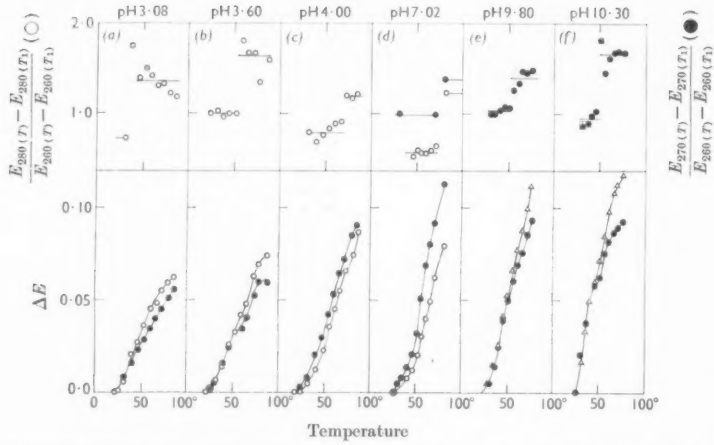

Fig. 4. Thermal denaturation of rabbit reticulocyte ribosomal RNA in 0·1 M-sodium phosphate buffers of different pH values. (*a*) At 19·9°, $E_{260}=0{\cdot}352$ and $E_{280}=0{\cdot}218$; for the lower-melting range $T_1=19{\cdot}9°$, otherwise $T_1=31{\cdot}3°$. (*b*) At 20·9°, $E_{260}=0{\cdot}345$ and $E_{280}=0{\cdot}187$; $T_1=20{\cdot}9°$ for the lower-melting range, otherwise $T_1=52{\cdot}8°$. (*c*) At 17·9°, $E_{260}=0{\cdot}343$ and $E_{280}=0{\cdot}172$; $T_1=23{\cdot}7°$ over the range 23·7–66·6°; otherwise $T_1=66{\cdot}6°$. (*d*) At 25°, $E_{260}=0{\cdot}71$ and $E_{280}=0{\cdot}31$; $T_1=25°$ for the lower-melting range, otherwise $T_1=70°$. (*e*) At 25°, $E_{260}=0{\cdot}876$ and $E_{270}=0{\cdot}669$; $T_1=25°$ for the lower-melting range, otherwise $T_1=50°$. (*f*) At 25°, $E_{260}=0{\cdot}876$ and $E_{270}=6{\cdot}707$; $T_1=25°$ for the lower-melting range, otherwise $T_1=45°$.

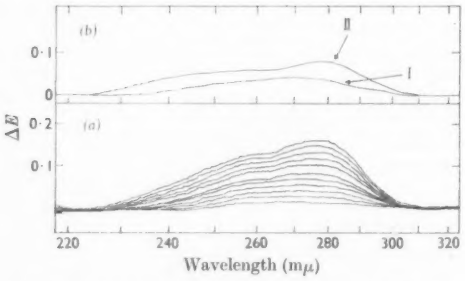

Fig. 5. Denaturation spectrum ($E_{(T)}-E_{(25)}$) of reticulocyte ribosomal RNA in 0·1 M-sodium phosphate buffer, pH 3·6. (*a*) T = 25, 30, 35, 40, 45, 50, 55, 60, 65, 70 and 75° successively. (*b*) Curve I, $E_{(40)}-E_{(25)}$; curve II, $E_{(75)}-E_{(50)}$. At 25°, $E_{260}=0{\cdot}850$.

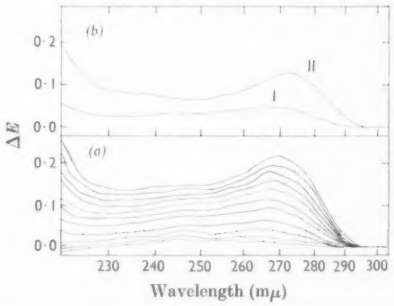

Fig. 6. Denaturation spectrum ($E_{(T)}-E_{(25)}$) of reticulocyte ribosomal RNA in 0·1 M-sodium phosphate buffer, pH 9·80. (*a*) $T=25$, 30, 35, 40, 45, 50, 55, 60, 65, 70 and 75° successively. (*b*) Curve I, $E_{(40)}-E_{(30)}$; curve II, $E_{(75)}-E_{(40)}$. At 25°, $E_{260}=0{\cdot}876$.

denaturation spectra are consistent with the denaturation of complementary base pairs in alkaline solutions (eqns. 17 and 18).

In more alkaline solutions, pH 10·32, secondary structure was less stable than at pH 9·80. The dependence of ΔE_{260} and ΔE_{270} on temperature was again biphasic (Fig. 4*f*). The difference spectra are given in Fig. 7: curve I in Fig. 7(*b*), which was characteristic of denaturation over the range 25–45°, was found to have a maximum at about 263 mμ ($\Delta E_{270}/\Delta E_{260}$ 1·0), and curve II in Fig. 7(*b*) had the same features as curve II in Fig. 6(*b*), although the $\Delta E_{270}/\Delta E_{260}$ value of 1·7 was somewhat higher. The difference spectra observed on heating both acidic and alkaline solutions of RNA are consistent with the denaturation of complementary base pairs.

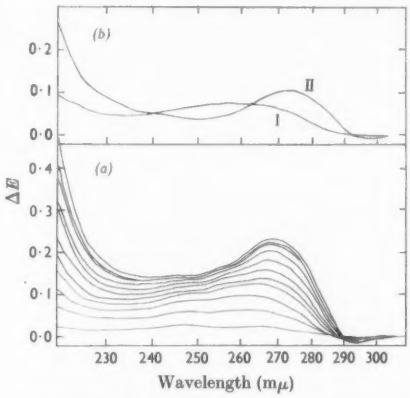

Fig. 7. Denaturation spectrum ($E_{(T)}-E_{(25)}$) of reticulocyte ribosomal RNA in 0·1 M-sodium phosphate buffer, pH 10·32. (*a*) $T = 25$, 30, 35, 40, 45, 50, 55, 60, 65, 70 and 75° successively. (*b*) Curve I, $E_{(35)}-E_{(25)}$; curve II, $E_{(75)}-E_{(45)}$. At 25°, $E_{260} = 0{\cdot}876$.

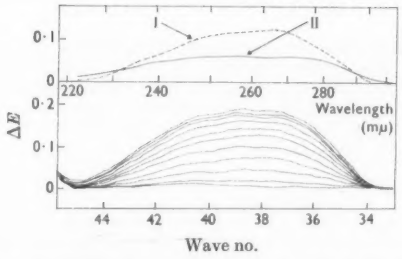

Fig. 8. Denaturation spectrum ($E_{(T)}-E_{(25)}$) of *E. coli* RNA in 5 mM-sodium phosphate buffer, pH 7·00. (*a*) $T = 25$, 30, 35, 40, 45, 50, 55, 60, 65, 70 and 75° successively. (*b*) Curve I, $E_{(50)}-E_{(30)}$; curve II, $E_{(75)}-E_{(50)}$. At 25°, $E_{260} = 0{\cdot}876$.

Unfractionated RNA from whole ribosomes was used in these experiments so that the results observed reflect principally the properties of the faster-sedimenting component. However, preliminary experiments showed that both the 16 s and 30 s components of RNA isolated from the fractionated ribosomal sub-units (prepared by titration with EDTA) (Arnstein, Cox, Gould & Potter, 1965) after fractionation had similar denaturation spectra in 0·5 mM-sodium phosphate buffer, pH 7. The melting ranges of the two species of helical segments were not identical and probably reflect the difference in the nucleotide composition of the two components (H. Gould, H. R. V. Arnstein & R. A. Cox, unpublished work; cf. Montagnier & Bellamy, 1964).

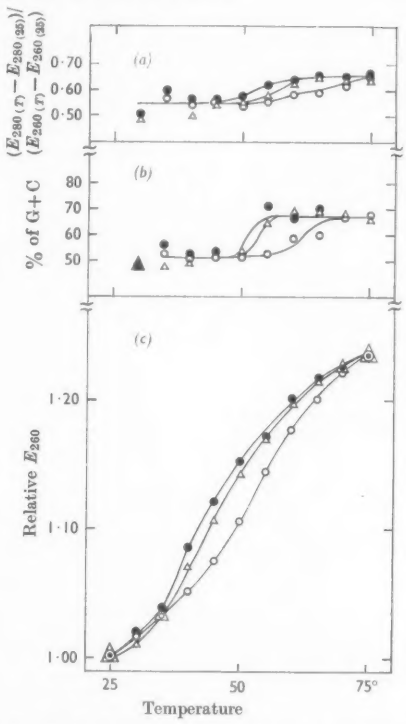

Fig. 9. Effect of electrolyte concentration on the thermal stability of the secondary structure of *E. coli* RNA. (*a*) $(E_{280(T)}-E_{280(25)})/(E_{260(T)}-E_{260(25)})$. (*b*) % of G+C in helical segments denaturing at the temperature indicated, calculated according to the procedure given in the legend to Fig. 3. (*c*) Dependence of $E_{260(T)}/E_{260(25)}$ on temperature. In each Figure ●, △ and ○ refer respectively to 0·5 mM-, 0·01 M- and 0·10 M-sodium phosphate buffer, pH 7·0.

Ribosomal RNA from E. coli. The experiments described above were repeated with RNA from *E. coli* ribosomes. The difference spectra obtained in 0·5 mM-sodium phosphate buffer, pH 7·0, are given in Fig. 8. Two distinct species could be discerned. In the range 30–50° the difference spectra ($E_{(T_2)}-E_{(T_1)}$) were characterized (curve I in Fig. 8*b*) by a maximum at about 267 mμ and a $\Delta E_{280}/\Delta E_{260}$ value of 0·55. In contrast, above 50° values of E in the range 240–280 mμ were similar to one another ($\Delta E_{280}/\Delta E_{260}$ 0·8).

Of the base pairs in helical segments denaturing over the range 30–50° the fraction of G+C was estimated as $50 \pm 4\%$ from eqn. (5), whereas helical segments melting above 55° were richer ($67 \pm 4\%$) in G+C.

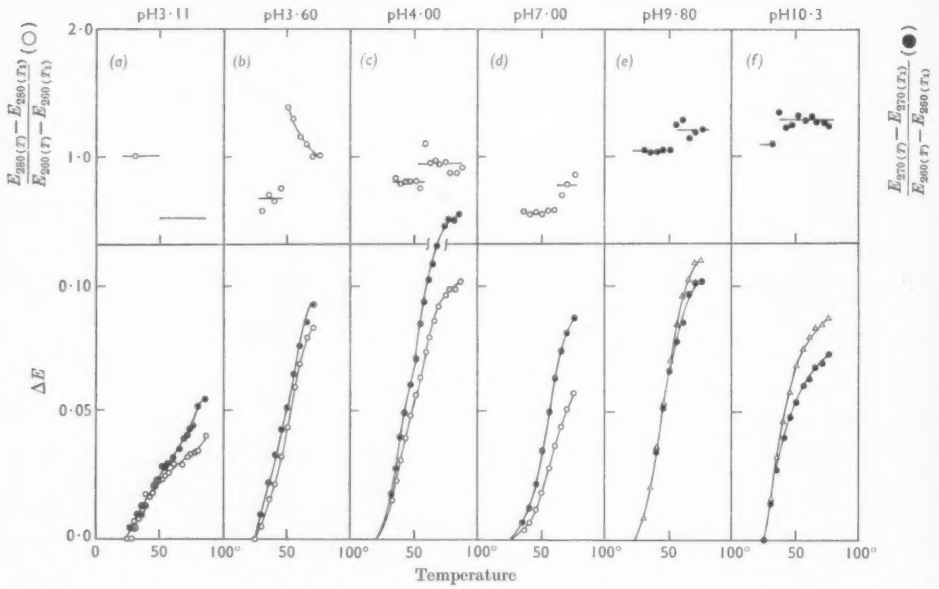

Fig. 10. Thermal denaturation of *E. coli* RNA in 0·1 M-sodium phosphate buffers at different pH values. (*a*) At 23·5°, $E_{260} = 0{\cdot}590$ and $E_{280} = 0{\cdot}304$; for the lower-melting phase $T_1 = 23{\cdot}5°$, otherwise $T_1 = 51{\cdot}7°$. (*b*) At 25°, $E_{260} = 0{\cdot}581$ and $E_{280} = 0{\cdot}289$: for the lower-melting phase $T_1 = 25°$, otherwise $T_1 = 45°$. (*c*) At 21°, $E_{260} = 0{\cdot}495$ and $E_{280} = 0{\cdot}233$; for the lower-melting phase $T_1 = 21°$, otherwise $T_1 = 54°$. (*d*) At 25°, $E_{260} = 0{\cdot}892$ and $E_{280} = 0{\cdot}395$; for the lower-melting phase $T_1 = 25°$, otherwise $T_1 = 60°$. (*e*) At 25°, $E_{260} = 0{\cdot}907$ and $E_{270} = 0{\cdot}687$; $T_1 = 25°$ or 45°. (*f*) At 25°, $E_{260} = 0{\cdot}927$ and $E_{270} = 0{\cdot}720$; $T_1 = 30°$ except over the range 25–30° when $T_1 = 25°$.

The relative increase in ΔE_{260} found over the range 25–75° is given in Fig. 9(*c*). The increase of about 17% (T_m approx. 40°) over the range 25–50° reflects the denaturation of helical segments of about 50% of G+C. The increase in E_{260} above 55° is attributed to the 'melting' of domains richer in G+C.

Increasing the concentration of sodium phosphate to 10 mM did not affect the temperature range over which denaturation took place (Fig. 9c), probably because of traces of bivalent cations in the stock RNA solution. However, increasing the electrolyte concentration to 0·1 M led to an increase in the stability of helical secondary structure. The difference curves obtained on denaturation were the same as those presented in Fig. 8 but higher temperatures were required to bring about the same transitions, e.g. segments of about 50% of G+C denatured over the range 30–60° (cf. 24–50° in 5 mM solutions).

The denaturation spectrum was hardly affected by the pH of the solution in the range pH 4·5–8·0. However, at pH 4·00 denaturation took place at slightly lower temperatures. Moreover, the dependence of ΔE_{260} and ΔE_{280} on temperature were both clearly biphasic (Fig. 10c) and an inflexion was noted at 55°. Over the range 25–55° the denaturation spectrum was skewed towards longer wavelengths (curve I in Fig. 11*b*) so that a $\Delta E_{280}/\Delta E_{260}$ value of 0·80 was found (cf. 0·55 at pH 7), and the melting range was slightly displaced towards lower temperatures. Above 55° the denaturation spectrum was further skewed towards longer wavelengths ($\Delta E_{280}/\Delta E_{260}$ 1·0).

At pH 3·60 the melting range was further displaced so that part of the transition from helical to amorphous forms had already taken place at 25° (Fig. 10*b*). Increasing the temperature from 25 to 87° led to differences in the spectrum consistent with the denaturation of two distinct species of helical segments as reported above; the species melting to an amorphous form below 45° was characterized by $\Delta E_{280}/\Delta E_{260}$ 0·85, and above 45° $\Delta E_{280}/\Delta E_{260}$ was higher and decreased from 1·4 at 50° to 1·0 at 75° (see eqn. 15).

In more acidic solutions (pH 3·11) denaturation

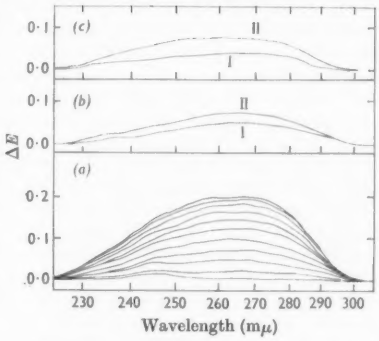

Fig. 11. Denaturation spectra of *E. coli* RNA in 0·1 M-sodium phosphate buffer, pH 4·06. (*a*) $T = 25$, 30, 35, 40, 45, 50, 55, 60, 65, 70 and 75° successively. (*b*) Curve I, $E_{(40)} - E_{(30)}$; curve II, $E_{(45)} - E_{(30)}$. (*c*) Curve I, $E_{(65)} - E_{(55)}$; curve II, $E_{(75)} - E_{(50)}$. At 25°, $E_{260} = 0{\cdot}900$.

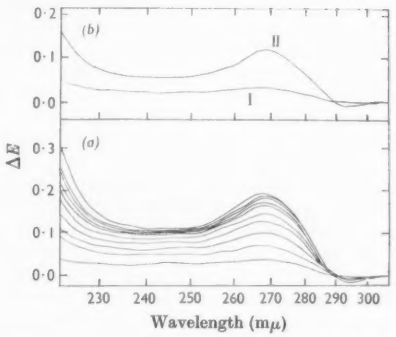

Fig. 13. Denaturation spectra of *E. coli* RNA in 0·1 M-sodium phosphate buffer, pH 10·30. (*a*) $T = 25$, 30, 35, 40, 45, 50, 55, 60, 65, 70 and 75° (the differences in curves for 65 and 70° were identical). (*b*) Curve I, $E_{(30)} - E_{(25)}$; curve II, $E_{(70)} - E_{(30)}$. At 25°, $E_{260} = 0{\cdot}927$ and $E_{270} = 0{\cdot}720$.

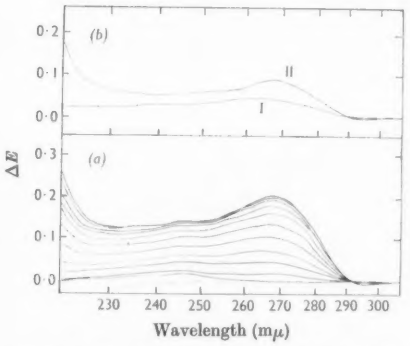

Fig. 12. Denaturation spectra of *E. coli* RNA in 0·1 M-sodium phosphate buffer, pH 9·80. (*a*) $T = 25$, 30, 35, 40, 45, 50, 55, 60, 65, 70 and 75° successively. (*b*) Curve I, $E_{(35)} - E_{(25)}$; curve II, $E_{(65)} - E_{(45)}$. At 25°, $E_{260} = 0{\cdot}907$ and $E_{270} = 0{\cdot}687$.

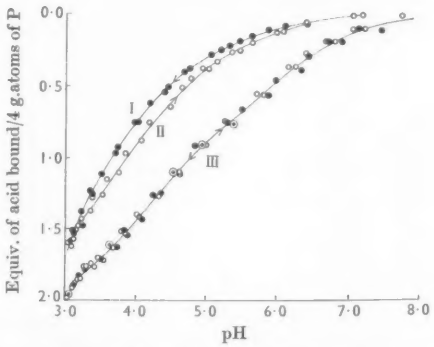

Fig. 14. Titration of reticulocyte ribosomal RNA in 0·1 M-NaCl at 25°. Curve I, ●, titration with acid from pH 7. Curve II, ○, titration with alkali from pH 3·0. Curve III, RNA fragments: ●, titration with acid from pH 7; ○, titration with alkali from pH 3 (⊙, coincident ● and ○).

took place at still lower temperatures and only the species richer in G + C persisted at 25° ($\Delta E_{280}/\Delta E_{260}$ 1·0). Above 50° a $\Delta E_{280}/\Delta E_{260}$ value of 0·5 was observed (Fig. 10*a*). Previously, evidence has been presented (Cox & Littauer, 1963) suggesting that at this pH the changes in the spectrum noticed above 55° are due to the denaturation of acid-stable secondary structure.

When the pH was raised from 7 to 9·80 denaturation was observed at lower temperatures (Figs. 10 and 12). Denaturation in the range 25–50° was characterized by the difference spectrum shown by curve I in Fig. 12(*b*), the value of $\Delta E_{270}/\Delta E_{260}$ being 1·05. The difference spectrum shown by curve II in Fig. 11(*b*) was observed on denaturation above 50° and has a well-defined maximum at about 267 mμ, as would be expected on denaturation of G + C-rich segments: the value of 1·2 was found for $\Delta E_{270}/\Delta E_{260}$.

At pH 10·30 the thermal stability of secondary structure was decreased still further, and for helical segments of about 50% of G + C part of the transition to an amorphous form had already taken place at 25° and was complete at 35° (Fig. 10*f*). The

difference spectra obtained are given in Fig. 13. The difference in the spectrum brought about by increasing the temperature from 25 to 30° (curve I in Fig. 12*b*) is comparable with curve I in Fig. 11(*b*). Increasing the temperature over the range 30–78° led to a more pronounced increase in E_{270} ($\Delta E_{270}/\Delta E_{260}$ 1·3; cf. 1·2 at pH 9·80), as shown by curve II in Fig. 13(*b*).

It is concluded that the changes in the spectrum of *E. coli* ribosomal RNA on denaturation, like those of reticulocyte ribosomal RNA, are entirely consistent with the denaturation of complementary base pairs. Moreover, there appear to be two principal species of helical segments, one having a nucleotide composition of about 50% of G + C, the other having about 67% of G + C.

Preliminary experiments with RNA from yeast showed that its secondary structure was essentially similar to that of *E. coli* RNA and reticulocyte ribosomal RNA although the nucleotide composition of the low- and high-melting phases were similar, about 55% of G + C and 60% of G + C respectively. The melting range was the same for both the crystallizable fragments of mol.wt. 14800 (W. B. Gratzer, unpublished work; quoted by Spencer & Poole, 1965) and higher-molecular-weight RNA, indicating that decreasing the mol.wt. to about 15000 did not appreciably affect helical secondary structure.

Electrometric titration studies. Reticulocyte ribosomal RNA (4mg./ml.) in 0·1 M-sodium chloride was treated at 25° with hydrochloric acid from pH 7 to pH 3·8 (curve I in Fig. 14), and the same curve was followed on titration with alkali from pH 3·8 to 7·0. However, after titration to more acidic pH values, e.g. pH 3·0, a different curve (II in Fig. 14) was followed on titration with alkali (cf. titration of rat-liver ribosomal RNA; Cox, 1963*a*). Curve I

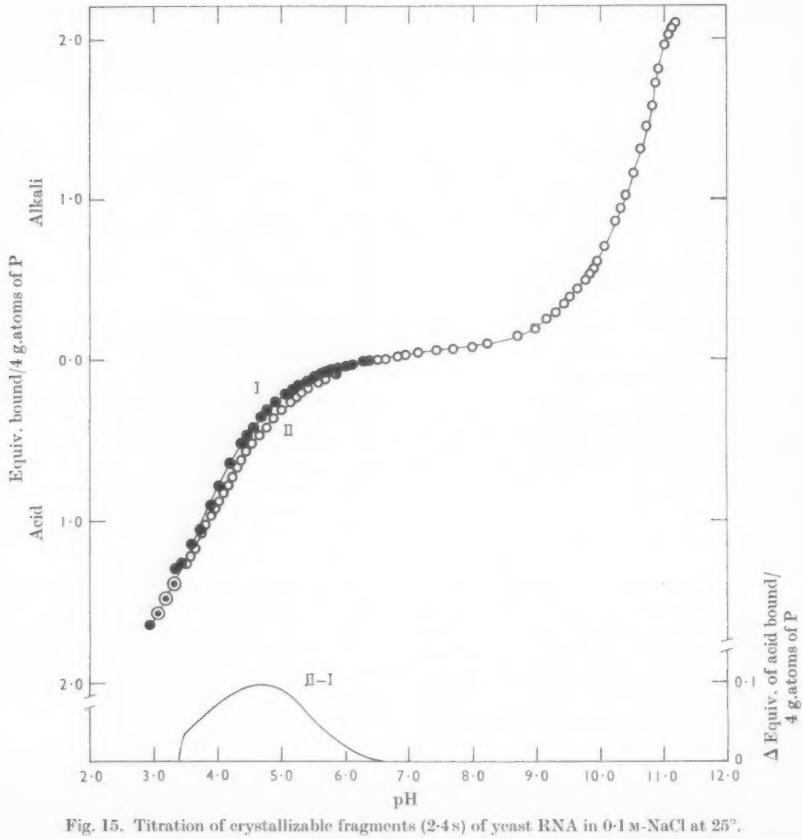

Fig. 15. Titration of crystallizable fragments (2·4 s) of yeast RNA in 0·1 M-NaCl at 25°.

in Fig. 14 was subsequently followed on titration with acid from pH 7, and curves I and II in Fig. 14 were accurately reproduced on successive titration cycles.

The titration properties of reticulocyte ribosomal RNA degraded at pH 13 by the method of Spencer & Poole (1965) were found to be essentially independent of molecular size provided that the fragments in 0·1 M-sodium phosphate buffer, pH 7, were characterized by $S_{20,w}$ values greater than about 2S. However, 1S fragments (curve III in Fig. 14) titrated over a higher pH range, as would be expected if secondary structure were absent. Moreover, the titration curve was reversible.

High-molecular-weight RNA from yeast as well as crystallizable (2·4S) fragments were also titrated. As reported above for reticulocyte RNA, hysteresis was observed over the range pH 3–7. Titration with acid from pH 7 to pH 4·0 followed the same course as titration with alkali from pH 4·0 to pH 7·0 (curve I in Fig. 15). A different curve (II in Fig. 15) was followed on titration with alkali from pH 3·6. In this case, too, curves I and II, which were the same for both crystallizable fragments and high-molecular-weight RNA, were reproduced on repeated titration cycles.

Hysteresis was also noted on titration of TYMV RNA in 0·1 M-sodium chloride at 25° (Fig. 16*a*) but the difference in equiv. of acid bound/4 g.atoms of P at a particular pH value along curves I and II was never more than 0·06 compared with 0·12 for yeast RNA and 0·16 for reticulocyte RNA.

The dependence of E_{260} on temperature at different pH values is given in Fig. 16(*b*). It is evident that the stability of the secondary structure of TYMV RNA decreased as the pH decreased. However, the persistence of a small increment in E_{260} over the range 25–50° at pH 2·80 is attributed to the denaturation of secondary structure that is stable in acidic but not in neutral solutions at 25° (cf. *E. coli* RNA; Cox & Littauer, 1963).

Transfer RNA from *E. coli* that was titrated over the range pH 3·0–7·0 had the same general features (Fig. 17) as TYMV RNA (Fig. 16). Although hysteresis was again observed, the difference in the equivalents of acid bound/4 g.atoms of P was never more than 0·05; this difference could be accounted for by the presence of about 25% of ribosomal RNA fragments in the preparation.

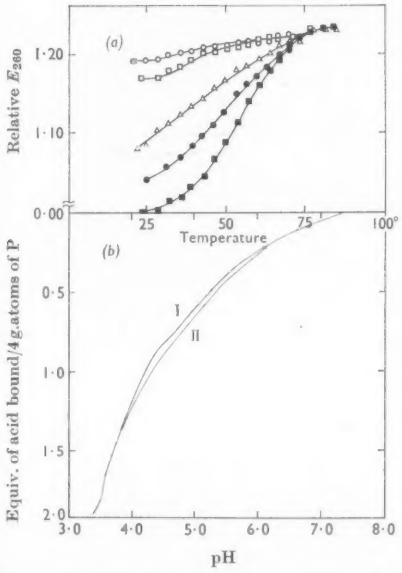

Fig. 16. (*a*) Effect of pH and temperature on E_{260} of TYMV RNA in 0·1 M-sodium phosphate buffer: ○, pH 2·80; □, pH 3·09; △, pH 3·60; ●, pH 4·01; ■, pH 4·42–7·0. (*b*) pH–titration curve for TYMV RNA in 0·1 M-NaCl at 25°.

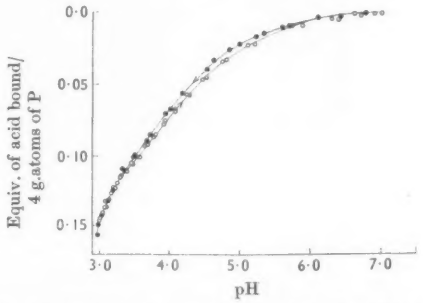

Fig. 17. pH–titration curve for *E. coli* transfer RNA in 0·1 M-NaCl at 25°.

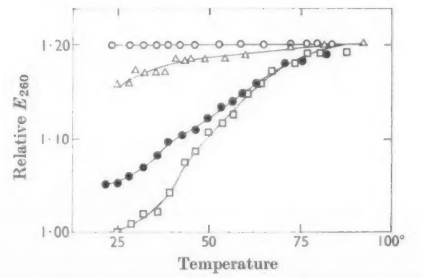

Fig. 18. Effect of pH and temperature on E_{260} of polyAGUC in 0·1 M-sodium phosphate buffer: ○, pH 2·80; △, pH 2·98; ●, pH 3·60; □, pH 4·01–7·0.

Insufficient polyAGUC was available for titration studies. However, it is possible to correlate hysteresis with the presence of acid-stable secondary structure as revealed by the dependence of E_{260} on temperature at different pH values. When polyAGUC was studied in this way (Fig. 18) it was found

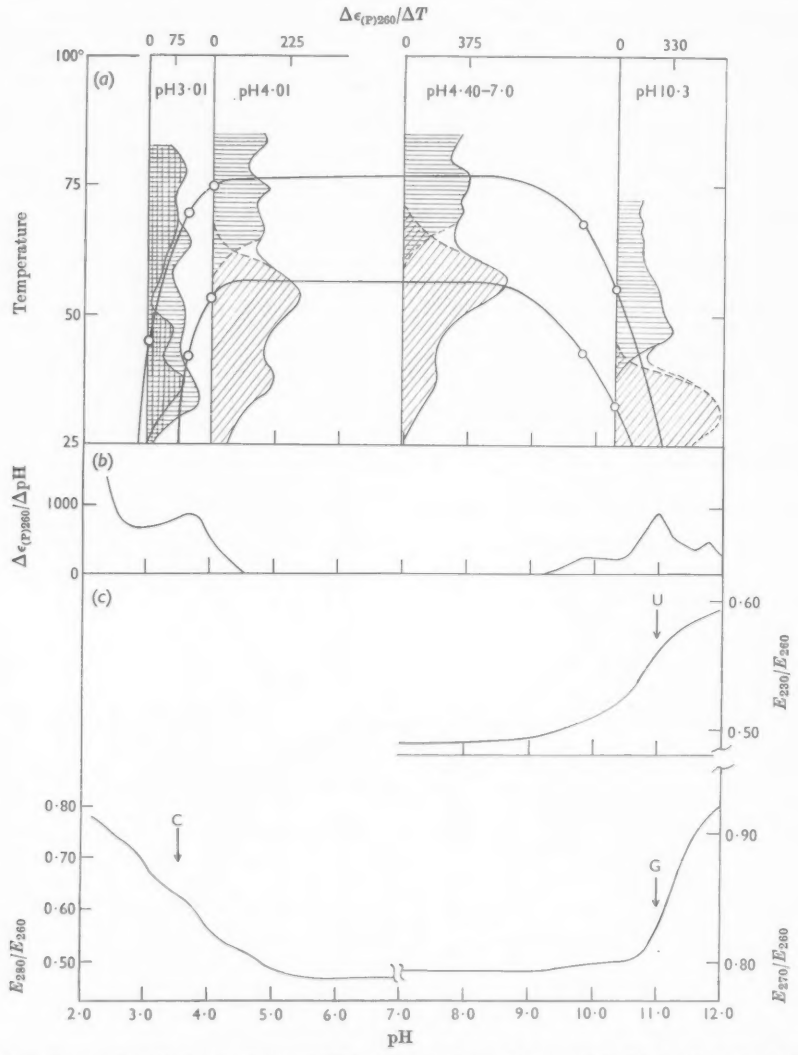

Fig. 19. Phase diagram for reticulocyte ribosomal RNA. (*a*) Dependence of T'_m on pH (in 0·1 M-sodium phosphate buffer). The ordinate $\Delta\epsilon_{260}/\Delta T$ indicates the relative proportion of base pairs in helical segments denaturing at a particular temperature. The area under the curve $\Delta\epsilon_{260}/\Delta T$ against temperature is equal to the total increase in E_{260}. ▨ denotes helical segments of about 50% of G+C; ▤ indicates helical segments of about 85% of G+C; ▥ denotes the melting profile of acid-stable secondary structure obtained by heating RNA in 0·01 M-sodium phosphate buffer, pH 3·0; ⊞ indicates overlapping ▥ and ▤. (*b*) $\Delta\epsilon_{260}/\Delta$pH derived from the data of Cox & Arnstein (1963). (*c*) Spectrophotometric titration data (Cox & Arnstein, 1963).

that decreasing the pH led to the elimination of secondary structure and no evidence for the presence of stable secondary structure in acidic solutions was obtained.

DISCUSSION

Nature of base-pairing in ribosomal RNA. There is increasing evidence that the hyperchromism of polynucleotides, which is due to the tendency of the base residues to stack one upon another, may be manifest when it is unlikely that double-helical structures are formed (e.g. Van Holde, Brahms & Michelson, 1965). Although a change in extinction is observed when a double-helical segment melts to an unfolded form it does not follow that an increase in extinction obtained by raising the temperature

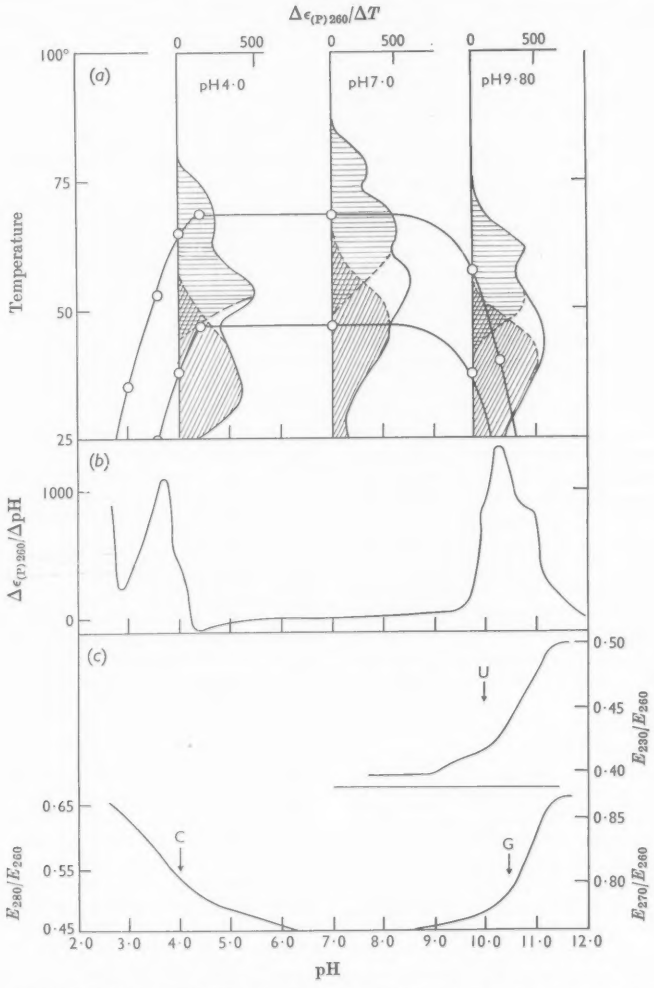

Fig. 20. Phase diagram for *E. coli* ribosomal RNA. (*a*) Dependence of T_m on pH (in 0·1 M-sodium phosphate buffer). The plot of $\Delta\epsilon_{260}/\Delta T$ has the same significance as in Fig. 19(*a*). ▨ and ▤ refer respectively to helical segments of about 50% and 67% of G+C. (*b*) $\Delta\epsilon_{260}/\Delta$pH, derived from the data of Cox & Littauer (1963). (*c*) Spectrophotometric titration data (Cox, 1961).

is always due to denaturation of double-helical secondary structure. It was therefore necessary to relate the denaturation spectra to at least one other independent property of double-helical structures: the acid–base properties of the base residues were chosen for this purpose.

The phase diagrams (Figs. 19 and 20) summarizing both the acid–base and denaturation studies are entirely consistent with the formation and denaturation, in a co-operative manner, of A--U and G--C base pairs. Both the denaturation spectra at different pH values and the spectrophotometric titration curves at about 25° are consistent with the pK values given in eqns. (7), (8), (17) and (18); the titration curves are hyper-sharp over the pH range where denaturation was observed; and the dependence of T_m on pH is analogous to that noted for polyAU (Cox, 1963*b*) and polyGC (Cox, 1966), i.e. T_m is decreased as the pH becomes more extreme as would be expected if the $NH_{(1)}$=$C_{(6)}$–NH_2 group of one residue were hydrogen-bonded to the $NH_{(1)}$–$CO_{(6)}$ group of another.

It is concluded that the secondary structure of ribosomal RNA at pH 7 is due principally, if not solely, to A--U and G--C base pairs since titration to the extreme of pH eliminates the structure stable at pH 7 (see also Cox, 1966). Ribosomal RNA appears to be amorphous at pH 12 at 25° since the difference in the spectrum observed in raising the pH from 7 to 12 is the sum of the denaturation spectrum obtained on heating to 87° at pH 7 and the difference spectra (Fig. 1) for the ionized and neutral forms of guanine and uracil residues (R. A. Cox, unpublished work; cf. Cox & Littauer, 1963).

It is not possible to show in the same way that secondary structure is entirely eliminated on titration to low pH values. Ribosomal RNA, on titration with acid from pH 7, behaves like the double-helical complex poly(A + U) formed between polyadenylate and polyuridylate (see below). At pH 7 the helical secondary structure is stabilized by interaction between A--U base pairs. As the pH is lowered this structure 'melts' and is replaced by the double-helical form of polyA that is stable in acidic but not neutral or alkaline solutions. The two forms do not attain thermodynamic equilibrium and the system exhibits hysteresis (Cox, 1963*a*): this is reflected in the phase diagram, which delineates the conditions of pH and temperature when hysteresis is observed. An analogous phase diagram was derived for *E. coli* ribosomal RNA (Fig. 21) from a knowledge of the range of pH over which hysteresis is found at different temperatures. It is also apparent from Figs. 19 and 20 that decreasing the pH leads to the elimination of A--U and G--C base pairs, and so it is concluded that decreasing the pH leads to the elimination of the secondary structure that is present at pH 7.

Nucleotide composition of helical segments. The nucleotide composition of helical segments of *E. coli* RNA and reticulocyte ribosomal RNA derived from the denaturation spectrum by using eqn. (5) agree with the values obtained from spectrophotometric titration data (Table 1). The fraction of base residues participating in helical secondary structure used to calculate the nucleotide composition of amorphous segments was deduced

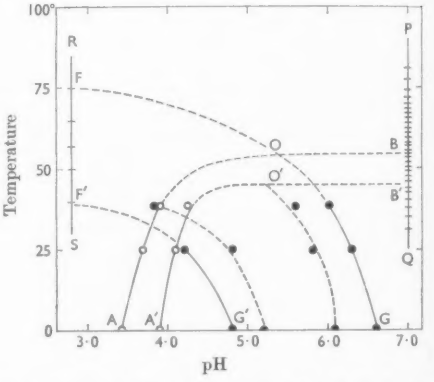

Fig. 21. Phase diagram for *E. coli* RNA in 0·1 M-NaCl. P–Q indicates the temperature range over which E_{260} increases at pH 7, the horizontal markers indicating the temperature interval over which E_{260} increased by 1%. ○–○ denotes the pH range over which, on titration from pH 7, the forward (cf. curve I in Fig. 14) and back titration (cf. curve II in Fig. 14) curves are generated: e.g. at 25° the base residues ionize reversibly on titration from pH 7 to pH 4 and back to pH 7·0, whereas after titration to more acidic pH values the forward and back titration curves are no longer identical, the difference being maximum at pH 3·6. Hence the zone AOB–A′O′B′ delineates the stability of the secondary structure that has to be disrupted to allow the formation of acid-stable structure. ●–● indicates the pH range, on titration with alkali from pH 3·0, over which the difference between the forward (in this case cf. curve II in Fig. 14) and back (cf. curve I in Fig. 14) titration curves is generated: e.g. at 25° the base residues ionize reversibly on titration from pH 3·0 to pH 4·1 and back to pH 3·0, whereas after titration to pH values in the range pH 4·1–4·7 a curve intermediate between curves I and II was followed on back titration with acid. A further change in the difference between forward and back titration curves was noticed when the pH was brought from pH 3 to in the range pH 5·8–6·3: when at pH 6·3 (or higher) curve I was followed on subsequent titration with acid. The zone FG–F′G′ delineates the stability of secondary structure that has to be disrupted to allow the original secondary structure that is stable at pH 7 to re-form. The curve R–S indicates the increase in E_{260} noted on heating *E. coli* RNA in 1 mM-NaCl, pH 3·0. The horizontal marker indicates an increase of 1% in E_{260}.

Table 1. *Nucleotide composition of helical and amorphous segments of ribosomal RNA*

Values in parentheses were derived from spectrophotometric titrations at 25° (cf. Cox, 1961). The values for reticulocyte RNA were derived from the spectrophotometric measurements of Cox & Arnstein (1963).

	Nucleotide composition (molar proportions)					
	Reticulocyte RNA			*E. coli* RNA		
	Total	Helical	Amorphous	Total	Helical	Amorphous
A	0·7	0·48 (—)	0·22 (—)	1·08	0·62 (0·58)	0·46 (0·50)
G	1·44	0·92 (1·3)	0·50 (0·14)	1·18	0·78 (0·8)	0·40 (0·35)
U	0·72	0·48 (0·42)	0·24 (0·3)	0·82	0·62 (0·53)	0·20 (0·35)
C	1·12	0·92 (1·12)	0·20 (0·1)	0·92	0·78 (0·72)	0·14 (0·2)
% of G+C ...	—	66	—	—	56	—

from the pH–titration curve (Cox, 1961; Cox & Littauer, 1963).

The spectrophotometric titration data also support the conclusion that there are two distinct species of helical segments that differ in nucleotide composition (cf. Cox, 1961). When reticulocyte RNA was titrated with acid at 25° secondary structure was unchanged over the range pH 4·0–7·0; the titration curve (curve I in Fig. 14) was reversible, and E_{280}/E_{260} increased over this range (Fig. 19) owing solely to the ionization of cytosine residues in amorphous segments. When the pH was further decreased, E_{280}/E_{260} increased, partly owing to the ionization of residues in helical segments (pK 3·1) and partly owing to an increase in the proportion of residues in amorphous segments as a result of denaturation. At pH 3·0, however, helical segments of 50% of G+C were denatured, the titration curve was no longer reversible (curve II in Fig. 14), and the secondary structure at this pH, indicated by the observation that not all cytosine residues were ionized, was identified from the denaturation spectrum as the segments richer in G+C.

Titration with alkali, at 25°, led first to the ionization of guanine and uracil residues in amorphous segments over the range pH 8·0–10·0; the proportion of uracil residues titrating over this range is greater than that of guanine residues. Continuing the titration to pH 11·0 led to the ionization of most of the uracil residues and to a smaller fraction of guanine residues as helical segments of 50% of G+C became denatured. Increasing the pH still further led to the ionization of the remaining guanine and uracil residues as helical segments richer in G+C denatured.

The same correspondence between the pH–titration properties and the denaturation spectra was observed for *E. coli* RNA (Fig. 20). Previously it was noted (Cox & Littauer, 1963) that titration from pH 7·0 to pH 4·5 at 25° led to no substantial change in E_{260}, indicating that secondary structure was unchanged over this range; the titration curve was reversible (cf. curve I in Fig. 14), and the increase in E_{280}/E_{260} was attributed to the ionization of cytosine residues in amorphous segments. Decreasing the pH further led to an increase in E_{260} with an inflexion at pH 3·3, and an increase in E_{280}/E_{260} to an extent consistent with the denaturation of helical segments poorer in G+C; a different integral titration curve (cf. curve II in Fig. 14) was followed on titration from pH 3·3. Helical segments richer in G+C were completely denatured at about pH 2·7.

Titration with alkali led first to the titration of guanine and uracil residues in amorphous segments over the range pH 8·0–9·8 and then to denaturation. The difference in the titration ranges of guanine and uracil residues was not as marked as for reticulocyte RNA, in accord with the inference that the difference in the nucleotide composition of the two species of helical segment is less for *E. coli* RNA.

The overall nucleotide composition of the helical segments ($f_{GC(total)}$) is due to fractions x and $1-x$ respectively of guanine plus cytosine residues in helical segments of low ($f_{GC(I)}$) and high ($f_{GC(II)}$) G--C content so that:

$$f_{GC(total)} = xf_{GC(I)} + (1-x)f_{GC(II)} \qquad (24)$$

In both *E. coli* RNA and reticulocyte RNA guanine and cytosine residues appear to be equally distributed among both species of helical segment: the species poorer in G+C appears to be shorter in chain length (see below), and probably occurs more frequently.

Chain length of helical segments. The double-helical segments of ribosomal RNA denature at lower temperature range than the highly polymerized RNA of the same average nucleotide composition, e.g. helical segments of about 50% of G+C 'melt' over the range 40–70° whereas the replicative form of encephalomyocarditis-virus RNA, which is believed to be double-stranded, has

a similar nucleotide composition but 'melts' at 96° (solvent, 0·15M-sodium chloride–0·015M-sodium citrate; Montagnier & Sanders, 1963). Studies with model compounds have shown that when N, the number of base pairs per helix, is small (about 20 or less) the melting temperature, T_m, depends on chain length. For ribosomal RNA N is probably also small.

OligoA of different chain lengths interacts with polyU forming double-helical structures [cf. poly(A+U)] having a well-defined melting range (Doty, 1961), and T_m and N are related by the equation (Lipsett, Heppel & Bradley, 1961):

$$T_m = 72 - (169/N) \quad (25)$$

and Lipsett (1964) found for poly(G+C) obtained by interaction of oligoG with polyC that:

$$T_m + 103 - (174/N) \quad (26)$$

The intercept but not the slope of eqns. (25) and (26) depends on the nucleotide composition of the double helix and in the general case:

$$T_m = T_{m(\infty)} - (172/N) \quad (27)$$

where $T_{m(\infty)}$ refers to the limiting value when N is large. Eqn. (27) was used to estimate N for the helical segments of reticulocyte ribosomal RNA and a range of 4–8 base pairs was calculated for segments of about 50% of G+C melting at 40–70°, and of 5–17 base pairs for the species of about 85% of G+C melting over the range 75–95° [in this case $T_{m(\infty)}$ was obtained by interpolation between the values of $T_{m(\infty)}$ given for poly(A+U) and poly(G+C) in eqns. (25) and (26)]. The helical segments of *E. coli* RNA were estimated to be within the range 3–8 base pairs, the average length of species of 50% of G+C being about 4 base pairs compared with an average of 6 base pairs calculated for the species richer in G+C. These estimates take no account of the possibility that T_m depends on nucleotide sequence as well as nucleotide composition, or that the helical segments may be imperfect (Fresco, Alberts & Doty, 1960; Bautz & Bautz, 1964).

The observation that 2s fragments (45 nucleotides long; Spencer *et al.* 1962) obtained by hydrolysing ribosomal RNA in the amorphous form have essentially the same helical secondary structure as the intact molecule (Figs. 14 and 15) shows that helical segments are shorter than the fragments, i.e. N is less than 22 base pairs. Once the average size of the fragments is less than the average size of helical segments, the original secondary structure will not re-form (e.g. curve III in Fig. 14, which characterizes 1s fragments) and a lower limit of helical size may be obtained, in this case about 6 base pairs.

Nucleotide sequence and hysteresis. The total hyperchromicity at 260mμ noted for *E. coli* transfer RNA, TYMV RNA and ribosomal RNA (Figs. 3, 9 and 16) is similar, indicating that the amount of secondary structure is the same. However, the hysteresis observed on titration over the range pH3–7 is greatest for ribosomal RNA irrespective of its nucleotide composition. This difference in properties is attributed to the presence in ribosomal RNA of a number of occasional clusters of adenine (or possibly cytosine) residues. A hypothetical polynucleotide chain that may form secondary structure having the average properties deduced for

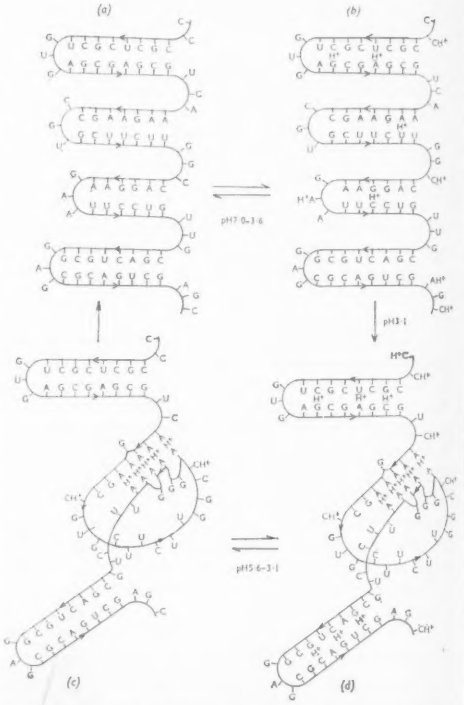

Fig. 22. Possible changes in the secondary structure of a polynucleotide having the same average nucleotide composition as reticulocyte ribosomal RNA due to titration from pH7 to pH3 and back to pH7. (*a*) Conformation stable at pH7. (*b*) Ionized form of (*a*) (see eqns. 7 and 8). (*c*) Helical segments poorer in G+C 'denature' on decreasing the pH below about 3·6, allowing the formation of acid-stable double-helical segments where the chains run parallel to one another (cf. Rich, Davies, Crick & Watson, 1961). (*d*) Apart from changes in the degree of ionization (*c*) persists until the pH is increased beyond about 5·6, when the acid-stable segments 'melt', so permitting (*a*) to re-form.

reticulocyte ribosomal RNA at pH 7 is represented in Fig. 22 (*a*). At 25°, over the range pH 4–7, secondary structure remains stable although adenine and cytosine residues ionize (Fig. 22*b*) according to eqns. (7) and (8). As the pH is further decreased the helical segments richer in A+U denature, permitting the formation of oligoA-like segments (Fig. 22*c*) that are stable in acidic but not in neutral or alkaline solutions (cf. Steiner & Beers, 1959); the polynucleotide chains run parallel to one another in this form (cf. Rich, Davies, Crick & Watson, 1961), and the p*K* of adenine residues is higher than that of AMP (Steiner & Beers, 1959). Thermodynamic equilibrium between the forms depicted in Figs. 22(*b*) and 22(*c*) is not attained (Cox, 1963*a*), so that subsequently raising the pH, e.g. to 4·5, does not alter the conformation, the original structure (Fig. 22*b*) cannot re-form, and the number of protons bound remains higher than at a comparable pH in forward titration (Fig. 22*b*). Once the pH is increased to a value where oligoA segments 'melt', the original structure re-forms, and the cycle may be repeated. There is no evidence that cytosine residues also tend to be found in clusters, although such clusters could also form oligoC segments (cf. polyC; Hartman & Rich, 1966) that are stable in acidic solutions and so contribute to hysteresis. *E. coli* transfer RNA and TYMV RNA appear to have fewer clusters of adenine or cytosine residues. Thus the hypothetical polynucleotide (Fig. 22*a*) appears to have the same denaturation and acid–base properties as reticulocyte ribosomal RNA.

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The Proteolysis of Immunoglobulin G with Long-Acting Thyroid-Stimulating Activity

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1. Proteolysis of immunoglobulin G with long-acting thyroid-stimulating activity with papain and pepsin shortened the duration of thyroid stimulation in mice prepared for the McKenzie (1958) bioassay. The time-course appeared to be related to molecular size. 2. The activity after proteolysis was recovered solely in the electrophoretically slow F_{ab}-fragment. 3. There was no immunological cross-reaction between pituitary thyroid-stimulating hormone and the active fragments of the long-acting thyroid stimulator despite their similar behaviour in the bioassay. 4. The implications of these findings are discussed.

Chemical and immunological studies on the LATS* detectable in the serum of some patients with thyrotoxicosis have shown it to be associated with IgG (Adams & Kennedy, 1962; McKenzie, 1962; Dorrington, 1964; Kriss, Pleshakov & Chien, 1964). Attempts to dissociate the thyroid-stimulating activity from IgG have been unsuccessful (McKenzie, 1962; Dorrington, 1964), suggesting that LATS activity may be an inherent property of specific IgG molecules.

Much information has been obtained relating the gross structure of IgG to serological activity, particularly in rabbit antibodies. Available evidence indicates that human IgG is essentially similar (Cohen & Porter, 1964). The four-polypeptide-chain structure proposed by Porter (1962) has been generally confirmed by subsequent work.

Analytical methods developed to study antibodies have been used in an attempt to determine the distribution of thyroid-stimulating activity in the peptide chains of LATS–IgG. Reduction of active IgG indicated that the heavy (γ) chains (Fleischman, Pain & Porter, 1962) carry the active portions of the molecule (Meek, Jones, Lewis & Vanderlaan, 1964; Dorrington, Munro & Carneiro, 1964). Separation of the peptide chains resulted in a variable loss of activity which probably accounts for discrepancies between the results from the two groups.

This paper describes results obtained with proteolytic enzymes used to degrade LATS–IgG in an attempt to locate the activity within the molecule.

* Abbreviations: LATS, long-acting thyroid stimulator; the nomenclature used for the immunoglobulins and their subfractions is that recommended by the World Health Organisation (see Cohen, 1965).

METHODS

Bioassay of thyroid-stimulating activity. The method of McKenzie (1958) was used with minor modifications (Major & Munro, 1962). To control assay conditions separate groups of mice (White Swiss; University of Sheffield Breeding Unit) were injected with 0·15 M-NaCl buffered with phosphate to pH 7·4, two doses of International Standard thyroid-stimulating hormone and two doses of a standard for LATS (Dorrington & Munro, 1964). Groups of five mice were used for each material tested. The volume injected intravenously was 0·5 ml. Blood samples (0·1 ml.) were withdrawn immediately before and 3 hr. and 10 hr. after injection.

The significance of the responses in the test groups has been tested against the control animals injected with 0·15 M-NaCl by using Student's t test and $P < 0·05$.

Isolation of IgG. Immunochemically pure IgG was obtained from active and control sera by precipitation with $(NH_4)_2SO_4$ followed by chromatography on DEAE-cellulose (Levy & Sober, 1960) with 0·01 M-phosphate, pH 6·6. The isolated IgG accounted for 10–12% of the total serum protein with $S_{20,w}$ 6·5–6·6 S.

Papain digestion of IgG. This was performed according to Porter (1959). Chromatographic analysis of the papain digest was effected by gradient elution from DEAE-cellulose (Edelman, Heremans, Heremans & Kunkel, 1960) at pH 7·8. The starting buffer was 5 mM-phosphate and the limit concentration 0·5 M-phosphate.

Protein determinations. Protein concentrations were determined by absorption at 276 mμ in a 1 cm. cell of an automatic recording spectrophotometer [Optica (U.K.) Ltd.].

Peptic digestion of IgG. Digestion with pepsin was carried out by the method of Nisonoff, Wissler, Lipman & Woernley (1960). In some experiments L-cysteine hydrochloride was

added after digestion to a concentration of 0·01 M and the mixture incubated at 37° for 2 hr.

Production of antisera. Antisera have been produced in rabbits (New Zealand Whites, 2·5–3·5 kg.) against LATS–IgG, papain F_{ab}-fragment, papain F_c-fragment and human pituitary thyroid-stimulating hormone.

The antigens were dissolved in 0·15 M-NaCl (1·0–5·0 mg./ml.), emulsified with an equal volume of Freund's complete adjuvant (Difco Corp.) and 0·1 ml. was injected intradermally at five sites. The injections were repeated 10 days later and the first bleeding (30–50 ml.) was performed after 2 weeks.

Neutralization studies. In the immunological cross-reaction studies the mixed antigen and antiserum were incubated for 1 hr. at 37° and then stored overnight at 4°. In all instances the proportion of antiserum was one-third of the total volume of the mixture. Before injection into assay animals any precipitate was removed by centrifugation at 900*g* for 10 min.

Ultracentrifuge analysis. Sedimentation-velocity measurements were performed at 59 780 rev./min. in a Beckman–Spinco model E ultracentrifuge equipped with schlieren optics. Samples were studied at 5–10 mg./ml. in 0·01 M-phosphate–0·15 M-NaCl buffer, pH 7·4, at 18–21° in a double-sector cell. Calculated values of the sedimentation coefficients were corrected to the viscosity of water at 20° ($S_{20,w}$).

RESULTS

Digestion of LATS–IgG with papain resulted in a striking change in the time-course of response in the McKenzie (1958) assay. The characteristic long-acting response of LATS–IgG was greatly shortened to give a maximal response at 3 hr., resembling pituitary thyroid-stimulating hormone (Table 1). This change accompanied the characteristic reduction in sedimentation rate of the digested IgG and separation of the antigenic determinants on immunoelectrophoresis (Edelman *et al.* 1960).

Chromatographic analysis of the papain digest on DEAE-cellulose separated the F_{ab}- and the F_c-fragments. With this system F_{ab} could be isolated free from F_c, as judged by immunoelectrophoresis. The short-acting thyroid-stimulating activity exhibited by the whole papain digest of LATS–IgG was localized entirely in F_{ab}; F_c was devoid of activity (Table 1).

After incubation of LATS–IgG with pepsin at pH 4·0 approximately two-thirds of the protein was recovered after dialysis. The sedimentation coefficient of the recovered protein was 4·9 s and when assayed the response to this component was long-acting, as the original IgG (Table 1). However, when this fragment was incubated with cysteine the sedimentation rate dropped further to 3·5 s and the assay response became short-acting, resembling the effect of papain (Table 1).

Since it was impossible to distinguish between the thyroid-stimulating activity of F_{ab} from LATS–IgG and pituitary thyroid-stimulating hormone from their bioassay responses, the relationship between the two was assessed by immunological cross-reaction studies (Table 2).

The long-acting response to LATS–IgG was completely annulled on incubation with anti-IgG, anti-F_{ab} and anti-F_c. It was not significantly affected by anti-(human thyroid-stimulating hormone) serum.

Incubation of the papain digest of LATS–IgG with anti-IgG and anti-F_{ab} abolished the short-acting response. However, anti-F_c and anti-

Table 1. *Effect of papain and pepsin on LATS–IgG as judged by the time-course of discharge of thyroidal* ^{131}I *in mice*

Each value represents the mean response of five animals. The type of response is indicated either as long-acting (L.A.), i.e. 10 hr. response > 3 hr. response, or short-acting (S.A.), i.e. 3 hr. response > 10 hr. response.

Sample	Mean blood radioactivity (% of initial value ± S.E.M.)		Type of response
	At 3 hr.	At 10 hr.	
LATS–IgG (10·0 mg./ml.)	740 ± 101	1436 ± 163	L.A.
Papain digest (10·0 mg./ml.)	396 ± 37	345 ± 29	S.A.
F_{ab}-fragment (10·0 mg./ml.)	745 ± 51	541 ± 64	S.A.
F_c-fragment (10·0 mg./ml.)	97* ± 6	93* ± 5	—
LATS–IgG (10·0 mg./ml.)	436 ± 108	967 ± 145	L.A.
Peptic digest (10·0 mg./ml.)	548 ± 67	671 ± 79	L.A.
Peptic digest + 0·01 M-cysteine (10·0 mg./ml.)	402 ± 31	276 ± 37	S.A.
Standard thyroid-stimulating hormone (0·06 milliunit/ml.)	510 ± 54	200 ± 25	S.A.

* Not significantly different from aq. 0·15 M-NaCl controls.

Table 2. *Cross-reaction studies between LATS–IgG, F_{ab}-fragment and human thyroid-stimulating hormone*

Values are for mean blood radioactivity (% of initial value ± S.E.M.) at 10 hr. for the first row and at 3 hr. for the second and third rows.

Antiserum ... ... Antigen	None	Anti-IgG	Anti-F_{ab}	Anti-F_c	Anti-(human thyroid-stimulating hormone)
LATS–IgG (1·0 mg/ml.)	1193 ± 250	181 ± 10*	148 ± 16*	113 ± 33*	1182 ± 87
Papain digest LATS–IgG (1·0 mg./ml.)	419 ± 59	136 ± 13*	77 ± 8*	359 ± 29	465 ± 32
Human pituitary thyroid-stimulating hormone (approx. 0·8 milliunit/ml.)	508 ± 72	403 ± 49	509 ± 64	491 ± 54	134 ± 10*

* Not significantly different from controls (33% antiserum in aq. 0·15 M-NaCl).

(human thyroid-stimulating hormone) sera were ineffective under identical conditions.

The assay response to human pituitary thyroid-stimulating hormone was only neutralized when incubated with the homologous antiserum.

DISCUSSION

The effect of papain and peptic proteolysis on the bioassay response to LATS–IgG confirms and extends the previous findings of Meek *et al.* (1964). These workers were able to isolate pieces I and II, using the nomenclature for rabbit IgG (Porter, 1959), although only piece I was active at the concentrations tested. It was suggested that the short-acting activity may have been due to thyroid-stimulating hormone bound to IgG, although there is considerable evidence that the two thyroid stimulators are distinct (Adams, 1965; Dorrington & Munro, 1965). Kriss *et al.* (1964) reported that papain destroyed all the activity of LATS–IgG although their modified McKenzie assay is insensitive to thyroid-stimulating hormone and probably also to a short-acting papain digest.

The results of the chromatographic and sedimentation-velocity studies on LATS–IgG agree with previous studies on human IgG with papain (Edelman *et al.* 1960; Franklin, 1960; Hsiao & Putnam, 1961) and on rabbit IgG with pepsin (Nisonoff *et al.* 1960).

Proteolysis of IgG has been shown to result in a decrease in molecular size (Charlwood, 1959; Noelken, Nelson, Buckley & Tanford, 1965) and it is reasonable to suppose that the change in assay response to LATS–IgG was associated with this decrease. This is supported by the finding that the 4·9 s fragment produced by pepsin (molecular size ~100000; Nisonoff *et al.* 1960) has a similar type of response to IgG. On reduction, however, the time-course of response became short-acting and the sedimentation rate decreased to 3·5 s.

Convincing evidence has been obtained that the thyroid-stimulating activity of LATS–IgG is associated exclusively with F_{ab}.

(1) Chromatographic analysis of the papain digest of LATS–IgG showed that only F_{ab} possessed activity. The isolated F_{ab}-fragments were immunochemically free of F_c.

(2) Biological activity in F_{ab} could only be neutralized by antisera containing antibodies to F_{ab} (i.e. anti-IgG and anti-F_{ab}). An antiserum to F_c was ineffective.

(3) Pepsin destroys F_c yet the activity remained and on reduction was associated with F'_{ab}. The F'_{ab}-fragments formed under these conditions appear to be similar to F_{ab} produced by papain (Mandy, Rivers & Nisonoff, 1961; Goodman & Gross, 1963).

The specific association of thyroid-stimulating activity with F_{ab} may be of significance, as the antibody-combining site of antigen-specific IgG is located on this fragment (Cohen & Porter, 1964). The disulphide bond-reduction studies of Meek *et al.* (1964) suggested that the A-chains were active when prepared from LATS–IgG. A combination of these two pieces of evidence implies that F_d (A-piece) carries the active sequence. However, more work needs to be done on isolated A- and B-chains in view of the discrepancies in the results obtained by Meek *et al.* (1964) and Dorrington *et al.* (1964).

The results presented in this paper provide circumstantial evidence that LATS activity is a property of peptide fragments within specific IgG molecules. On the basis of this evidence, however, it is not possible to decide whether LATS is an antibody.

An autoimmune aetiology for thyrotoxicosis has been suggested (Adams, 1965), involving the interaction of LATS–IgG with an unidentified thyroidal antigen. Various suggestions have been put forward as to the nature of this antigen. An intracellular thyroid-stimulating hormone binding

site has been implicated (Adams, 1965; Dorrington, 1964), possibly associated with the nucleus (Kriss *et al.* 1964) or some factor which normally inhibits thyroid metabolism (Dorrington, 1964; Kriss *et al.* 1964). However, in the last analysis proof of this hypothesis will depend on the characterization of a thyroidal antigen which initiates the synthesis of a specific IgG with LATS activity. The search for such an antigen is being actively pursued in several Laboratories.

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The Hatching Factor of the Potato-root Eelworm

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1. The hatching factor of the potato-root eelworm was concentrated from potato-root leachings by adsorption on charcoal. The crude material, extracted from charcoal with acetone, was purified by partition between ethyl acetate and M-potassium dihydrogen phosphate, and ethyl acetate and a solution of potassium metabisulphite (disulphite). Neutral material was removed by extraction from phosphate buffer at pH 7·8. The yield of purified material, after chromatography on silica, DEAE-cellulose and Perlon columns, was 18 μg./l. of potato-root leachings, and 0·04% by weight of the acetone-soluble material extracted from charcoal; 20% of the activity of the original potato-root leachings was recovered. 2. The purified material was a colourless gum. On paper chromatograms it gave a single weakly fluorescing spot, which reacted with 2,4-dinitrophenylhydrazine and aniline–xylose reagents. It was confirmed that the hatching factor is an acid, and it is inactivated at values above pH 9. Other properties were a ready oxidation by permanganate and reaction with certain carbonyl reagents. Colour tests for α- and β-unsaturated γ-lactones were negative. The hatching factor showed no light-absorption maxima at wavelengths above 210 mμ; absorption bands in the infrared region (670–4000 cm.$^{-1}$) are given. Our purified material, although a gum, gave a similar hatch to a 'crystalline' product at 640 times its concentration.

The potato-root eelworm (*Heterodera rostochiensis* Wollenweber) is stimulated to hatch from its cysts by diffusates from the roots of potato (*Solanum tuberosum* L.), tomato (*Lycopersicon esculentum* Mill.) and many other solanaceous plants. Williams (1958) found evidence of activity with plants of the genera *Atropa*, *Browallia*, *Datura*, *Nicotiana* and *Celsia*, but only the genus *Solanum* consistently gave diffusates of high activity. Extracts from all parts of potato plants, except the tubers (Janzen & van der Tuin, 1956; Carroll, Heyes, Johnson & Todd, 1958), also stimulate hatching.

In previous attempts to isolate the active substance the hatching factor in root leachings was concentrated by adsorption on charcoal or on ion-exchange columns, or by evaporation *in vacuo*. Although various methods have been tried for the purification of crude concentrates no satisfactory procedure has yet been reported.

Properties of various preparations of the hatching factor are described by Marrian, Russell, Todd & Waring (1949), Widdowson & Wiltshire (1958) and Hartwell, Dahlstrom & Neal (1960).

MATERIALS AND METHODS

Cyst stocks. *Heterodera rostochiensis* cysts from various sources were used. They were collected in the autumn and were stored dry for up to a year, in the dark and at room temperature.

Assay procedure. The cyst-hatching technique used for the bioassay was that of Fenwick & Widdowson (1958). Tests were usually continued for 3 weeks, but the larvae were removed weekly and counted after 1 and 3 weeks. Each set of samples for assay included the standard root-diffusate solution at six dilutions (1/1, 1/4, 1/16, 1/64, 1/256, 1/1024) and the test samples at a minimum of three dilutions (usually 1/4, 1/16, 1/64). Batches of 100 cysts in fivefold replication were used for each dilution, unless otherwise stated. The cumulative larval hatch from each set of replicates was plotted against a logarithmic dilution scale. A straight line was fitted by eye to the linear part of the curve and projected to meet the line for hatch in water. The point of intersection of these lines gave, on the log scale, a relative measure of the hatching factor present, i.e. log activity (L.A. value) of the solution (Fenwick, 1952).

Widdowson & Wiltshire (1958) related concentrations of active material in samples in terms of specific activities (sp. activity = antilog L.A./mg. dry wt./l.).

As far as possible, the concentration of the test samples was arranged to give levels of hatching within the linear part of the standard curve (larval emergence/dilution).

When few larvae emerged, the hatching curve was shallow and sloped, which made the interpretation of the tests more difficult. Improved hatching curves were obtained in these circumstances by increasing the duration of the tests and counting the larvae after immersion in the test solutions for 2 and 4 weeks.

A simplified assay, in which samples were used at a single dilution and the tests lasted for 1 week only, was

used when testing many samples, e.g. fractions from chromatographic columns.

Assay standards. (i) Potato-root leachings, collected in spring, were stored at 3° in the dark in a 50l. glass carboy, standardized (Fenwick, 1952) against the appropriate cyst population and then used as the reference standard for all hatching tests during the succeeding 12 months.

(ii) A solution of the factor in acetone was prepared with material obtained by treating potato-root leachings (40l.) with charcoal, and purifying the extracts by the procedure given. This concentrate, dissolved in acetone (40ml.) and stored at 0–3° in the dark, was used as an additional standard in assay tests (diluted 1/500 in water), for 6 consecutive weeks and at 14 weeks (seven tests in all). The diffusate before treatment had L.A. 2·5 and the acetone solution (40ml.) assayed at 0·05ml./50ml. of water, had L.A. 2·1, a recovery of approximately 40% of the original activity.

Silica columns. Silica columns were prepared with silica (A.R. grade, Mallinckrodt Chemical Works, New York, N.Y., U.S.A.) triturated with the appropriate buffer (0·3ml./0·5g. of silica). The treated silica was made into a slurry with the initial eluting solvent, poured into a chromatography tube (1·2cm. diam.) and the excess of solvent run off before use.

Ion-exchange materials. (*a*) A column (54cm.×4·0cm.) of De-Acidite FF (−16×50 mesh) was used when treating large volumes of potato-root leachings. Before use the column was washed with 3% NaOH (800ml.), then water (1·5l.), followed by 2N-acetic acid (800ml.) and water (2·0l.). (*b*) DEAE-cellulose powder (240g.) was washed with 0·2N-NaOH (5l.) and then with water until the eluate was neutral. (*c*) Small-scale columns. A column of DEAE-cellulose (8g.; 24cm.×2cm.) in the formate form was prepared by washing the powder first with 0·1N-NaOH (200ml.), then with water until the eluate was neutral. A 4N-formic acid solution (500ml.) in acetone–water (1:1, v/v) was passed through the column, followed by water until the eluate was neutral. Finally, the column was washed with acetone–water (1·1, v/v). Other DEAE-cellulose columns in the formate form were prepared similarly.

Perlon columns. Columns of Perlon powder (20g.; 18·0cm.×2·2cm.) were prepared for use by washing with 0·1N-NaOH (200ml.), then water until a neutral eluate was obtained, followed by 0·1N-HCl (200ml.) and water until free from acid. The Perlon (polycaprolactam) powder (Farbwerke Hoechst, Werk Bobingen, Germany) was a gift from Dr H. G. C. King, Pedology Department, Rothamsted.

Potato-root leachings. Potatoes (*Solanum tuberosum* L., variety Arran Banner) were grown in sandy loam in 6½in. (top diam.) flower pots, some in glasshouses and some in the open. The pots were kept on sloping asbestos-topped benches, and leached by first saturating the soil with tap water and then adding more (50–100ml.). Leaching was started within 7 days of the shoots emerging and was repeated on alternate days for 6 weeks. Leachings were also obtained from potato plants (about 40 per bed, 2·5m.×1·1m.×26cm.) grown in soil over a layer of shingle on asbestos-topped benches. The beds were leached on alternate days by spraying with water until 10l. of leachings was collected. With both methods the leachings drained into channels on the benches and were collected in pails and then transferred to 50l. glass carboys.

Preparations of crude concentrations of the factor. Of the three methods mainly used as the first treatment of the potato-root leachings, adsorption of the active material on charcoal was better than concentration by evaporation or adsorption on ion-exchange materials.

(*a*) Adsorption on charcoal. To the yellow–brown leachings (40l.) were added conc. HCl (30ml.) and decolorizing charcoal (120g.). The liquid was stirred thoroughly and left to stand for a day, when the clear colourless supernatant liquid was poured away. The charcoal was resuspended in the 2–3l. of remaining liquid, and the suspension filtered on a Buchner funnel. The charcoal was transferred to a beaker where it was washed with successive portions (4×300ml.) of acidified acetone (0·4ml. of conc. HCl/100ml. of acetone). At each washing the charcoal was stirred with the solvent to get a uniform suspension, which was then filtered. The combined washings were evaporated to dryness at 10–20mm. pressure in a rotating evaporator with an external bath temperature of 34–40°, to leave a mixture of organic and inorganic material (0·6–2·0g.).

(*b*) Concentration by evaporation. Potato-root leachings (20–26l.) were acidified with acetic acid (1ml./l.) and concentrated to 50/75ml. at 30–40° and 10–20mm. pressure. The pH was kept at about 5 by adding further acetic acid. The sludge obtained was brought to pH2 with conc. HCl, and extracted with ethyl acetate (40ml., 12×20ml.). Evaporation of the extracts *in vacuo* left a gum (0·3–0·8g.).

(*c*) Adsorption on De-Acidite FF. Potato-root leachings (20l.) were run through a column of De-Acidite FF (54cm.×4cm.) in the acetate form. The column was then washed with water (2 bed vol.), allowed to drain and eluted with a mixture of acetone, water and conc. HCl (700ml.:300ml.:35ml.), which gave a golden-yellow eluate. Elution was continued with acetone and water containing conc. HCl (700ml.:300ml.:9ml.) until the eluate was colourless. The combined eluates yielded a gum (0·5–2·0g.) when evaporated *in vacuo*.

(*d*) Adsorption on DEAE-cellulose. DEAE-cellulose (240g.) in the free base form was added to potato-root leachings (40l.) containing acetic acid (100ml.). The mixture was stirred for 30min. and the DEAE-cellulose recovered by filtration. The DEAE-cellulose was shaken with a mixture of acetone (700ml.), water (300ml.) and conc. HCl (40ml.). The resulting slurry was poured into a chromatography tube, and the solvent mixture run off. The column was further eluted with acetone–water (7:3, v/v; 1l.). The combined eluates yielded a gum (0·5–1·5g.) on evaporation *in vacuo*.

Purification of the crude concentrates. The residues obtained by the above procedures were submitted to the purification procedure outlined in Scheme 1.

All extractions were made in 100ml. centrifuge bottles so that any emulsions that formed could be broken by centrifugation. As the hatching factor is readily destroyed by alkali, solutions were kept below pH8. Solvents were removed by evaporation *in vacuo* in a rotary evaporator with an external bath temperature of 30°. Active material was stored in acetone solution at −10°.

The crude residue (4–14g.) was shaken with ethyl acetate (20ml.) and M-KH_2PO_4 (15ml.). The upper, ethyl acetate, phase was removed, and extraction continued with fresh portions of ethyl acetate (12×20ml.). After removal of solvent from the combined extracts, the residue (A) was taken up in ethyl acetate (20ml.) and the solution shaken with potassium metabisulphite (4ml. of saturated

solution, 4ml. of water). The ethyl acetate layer was removed and the aqueous phase extracted with more ethyl acetate (12×20ml.). Removal of ethyl acetate left a resin (B) which was treated with 0·57M-K_2HPO_4 (20ml.). The pH of the solution was adjusted to pH7·8–8·0 by adding 5M-K_2HPO_4 to the stirred liquid. The solution was extracted with ether (5×20ml.), then ether–ethyl acetate (1:1, v/v; 8×20ml.). The residual aqueous phase was brought to pH2 with conc. HCl and the active material extracted with ethyl acetate (12×20ml.). Evaporation of the extracts left a yellow gum (C) which was further purified by chromatography.

A solution of the resin (C) in acetone (100ml.) was run through a DEAE-cellulose (formate form) column (16g.; 17cm.×4·0cm.). Elution with 1mM-formic acid in acetone was continued until the yellow eluate gave way to colourless fractions (total volume of eluate 1800ml.). Yellow–brown impurity was left adsorbed on the column, whereas most of the hatching factor was contained in the acetone eluate. Further elution of the column with 0·5N-formic acid in acetone brought off a small amount of a more impure but active material. Overloading of the column showed either by adsorbed yellow–brown material occurring throughout the column, or, with too much inorganic acid, by the upper part of the column failing to retain yellow–brown material; when either happened, the 1mM-formic acid eluate was evaporated to dryness and the process repeated with a fresh column.

The acetone solution was evaporated to dryness and the residue (D) dissolved in chloroform (20ml.). Silica (1·0g. treated with phosphate buffer, pH2; 0·6ml.) was added to the chloroform solution, and the solvent removed *in vacuo*. The yellow powder of gum absorbed on silica was thoroughly mixed and added to the top of a column (3cm.×1cm.) of silica (2g. treated with phosphate buffer, pH2; 1·2ml.) made up with light petroleum (b.p. 60–80°)–chloroform (2:1, v/v). The residues of the gum adsorbed on silica were treated with chloroform (20ml.) and with more buffered silica (0·2g.). The solvent was removed and the powder added to the top of the column.

The column was eluted successively with the following mixtures (v/v) of light petroleum (b.p. 60–80°) and chloroform: 2:1 (30ml.), 1:1 (30ml.), 2:3 (30ml.), and then with chloroform (200ml.). Each eluent brought off a yellow fraction. A yellow band remaining on the column after elution with chloroform was removed with ethyl acetate. The bulk of the hatching factor was in the chloroform eluate, but there were small amounts in the 1:1, 2:3 and ethyl acetate eluates.

Evaporation of the chloroform eluate left a clear pale-yellow gum (E), which was triturated with successive portions of diethyl ether (10×20ml.). Most of the hatching factor passed into solution. The ethereal extracts were run through a short column of silica (1g.; treated with M-KH_2PO_4, 0·6ml.) and followed by a 20ml. of ether. The pale-yellow eluate was active and some yellow–brown impurity was retained on the column.

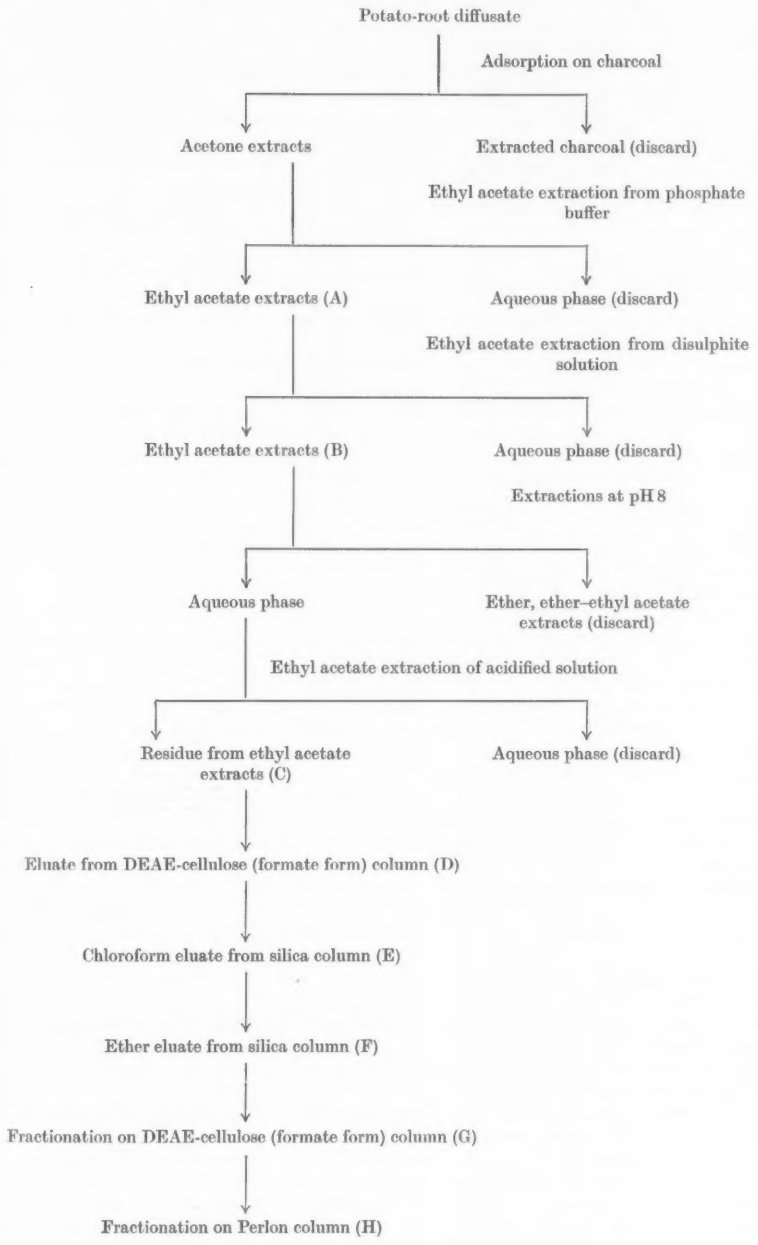
The ether eluate was evaporated and the residue taken up in sodium-dried ether (100ml.) and filtered to separate the soluble active material from small amounts of inorganic impurity (see below). After removal of ether the residue (F) was added to DEAE-cellulose (formate form) column (8g.; 21cm.×2·2cm.) in successive portions of acetone–water (1:1, v/v; 10×10ml.). Twelve 10ml. fractions of 1:1 eluate were first collected, and then 40×10ml. with 1mM-formic acid (in acetone–water, 1:1, v/v). The hatching factor was distributed through the 1mM-formic acid fractions and further elution with more concentrated formic acid brought off only very little more.

It was important with the above column of DEAE-cellulose (*a*) that it initially was free from formic acid and (*b*) that material applied to it was free from traces of mineral acid, phosphate buffer etc. Otherwise the initial eluate of acetone–water (1:1, v/v) contained active material, as it did when the acidic impurities eluted with acetone–water were excessive. Fractions from each elution were therefore assayed. Some indication of the course of the elution of impurities, particularly of those eluted with acetone–water (1:1, v/v), was obtained by reading the light-absorption of the various fractions at 333mμ against water. Absorption at this wavelength by the hatching factor is negligible.

The most active fractions of the 1mM-formic acid eluate were combined and evaporated to dryness. The small amount of gum (G) left was treated seven times with 10ml. of water and each 10ml. portion was run on to a Perlon column (20g.; 18·0cm.×2·2cm.) made up with water. After the seven 10ml. fractions had been collected, the column was eluted with acetone–water (1:9, v/v) and 20×10ml. fractions were collected. Subsequent elution with acetone brought off inactive impurities. The active material was present in the acetone–water eluates. As with the eluates from the DEAE-cellulose column, the presence of active material in the fractions was detected by hatching tests, and elution of impurities could be followed from the absorption of the fractions at 333mμ (read against water). As before, fractions were combined according to their hatching activity. Evaporation of these eluates gave a colourless gum (H), which was immediately dissolved in either ether or acetone. This purified material was used as a starting material for chemical investigations.

Samples were taken for assay at stages (A), (B) and (C), and diluted so that the concentration was directly related to that of the parent root diffusate. Thus where the material was derived from 40l. of root diffusate, a 0·05ml. sample would be taken from a 40ml. acetone solution of (A), (B) or (C), and made up to 50ml. with water after neutralization with a dilute solution of calcium hydroxide. The complete assay for a batch of root diffusate included the parent root diffusate, the filtrate after charcoal adsorption, and samples (A), (B) and (C). Purification of the product after stage (C) was followed in separate bioassays. All samples obtained from fractions from a chromatographic column were assayed simultaneously, together with a sample of the starting material.

Paper chromatography. A descending one-dimensional technique was used with Whatman no. 1 paper. Solvent systems used were: (*a*) 0·1M-KH_2PO_4; (*b*) phenol–water (4:1, w/v) (as used by Massey & Neal, 1953); (*c*) M-NH_4Cl; (*d*) acetone–water (1:19, v/v) (Widdowson & Wiltshire, 1958); (*e*) butanan-2-one–water (3:50, v/v); (*f*) butan-1-ol–90% formic acid–water (10:3:10, by vol.; upper phase); (*g*) chloroform–2-methylbutan-2-ol–90% formic acid–water (136:24:3:80, by vol.; upper, i.e. aqueous, phase). Material purified to stage (C) from subsequent stages was made up in acetone solution (2–6mg./ml.), and applied (11×0·005ml.) to Whatman no. 1 paper. Development of the chromatograms was stopped when the solvent front had travelled


Potato-root diffusate
Adsorption on charcoal
Acetone extracts
Extracted charcoal (discard)
Ethyl acetate extraction from phosphate buffer
Ethyl acetate extracts (A)
Aqueous phase (discard)
Ethyl acetate extraction from disulphite solution
Ethyl acetate extracts (B)
Aqueous phase (discard)
Extractions at pH 8
Aqueous phase
Ether, ether–ethyl acetate extracts (discard)
Ethyl acetate extraction of acidified solution
Residue from ethyl acetate extracts (C)
Aqueous phase (discard)
Eluate from DEAE-cellulose (formate form) column (D)
Chloroform eluate from silica column (E)
Ether eluate from silica column (F)
Fractionation on DEAE-cellulose (formate form) column (G)
Fractionation on Perlon column (H)

Scheme 1. Flow sheet of the purification procedure.

12in. from the point of application of the sample. After drying at room temperature, the paper was cut into strips of equal R_F width (0·05 R_F unit), and each strip placed in a flask containing water (50ml.). The flask was shaken occasionally during the next 30min., and the solution then assayed in three- to five-fold replication at three dilutions. Paper chromatograms were examined for fluorescent material under ultraviolet light (Hanovia Ltd.) model 18 lamp. Other means of detection used were Brady's reagent, and for chromatograms developed with solvent systems (*b*), (*d*) and (*g*), the aniline–xylose reagent of Wood (1958).

RESULTS

Concentration of the hatching factor. Most of the active material in acidified potato-root leachings was removed by charcoal (3g./l.) and 31% was recovered from the charcoal in the acetone extract. Various other methods of treating the charcoal, such as hot extraction with acetone, elution with methanol or acetone–water mixtures, did not increase the recovery. A yield of 20% (average of ten experiments) of the activity of the original potato-root leachings was obtained by concentration by direct evaporation. Passage through a column of De-Acidite FF (acetate form) removed 95% of the activity from the potato-root leachings, and 23% (average of ten experiments) of the initial activity of the leachings was recovered from the column in the acid eluates. However, to do this required repeated elution with acid because the amount of hatching activity released tailed off very gradually.

Treatment of potato-root leachings with DEAE-cellulose absorbed only about 55% of the hatching factor. The amount recovered in the acetone eluate of the cellulose was 10% (average of four experiments) of the initial; the eluate end-point was much sharper than with De-Acidite FF.

Purification of crude concentrations. Ethyl acetate extracts hatching factor from aqueous solutions provided that these are acid and contain salt. In treatment of the active brown gum obtained by evaporating the acetone washings from charcoal, as shown in Scheme 1 to reach product (C), 90% of the material in the original gum was eliminated while retaining 85% of the hatching activity. The further procedures to get from product (C) to product (H) eliminated more than 98% of the material in product (C) while retaining about 75% of its hatching activity. In one experiment done with 280l. of potato-root leachings the residue obtained on evaporating the acetone washings of the charcoal weighed 12·7g., and products (A), (B) and (C) 4·6, 2·2 and 1·3g. The weights of later stages were 249mg. (D), 84mg. (E), 70mg. (F), 32mg. (G), 5mg. (H). Hence the yield of the final product (H), which had 20% of the hatching activity of the total initial activity, was equivalent to 18μg./l. of the starting material.

Properties of the hatching factor. The hatching factor dissolved readily in acetone, ethyl acetate, methanol or ethanol, less readily in ether, chloroform and water, and was insoluble in light petroleum, benzene or carbon tetrachloride. Our work confirmed that it was an acid and was rapidly inactivated at values above pH9.

A solution of the hatching factor in ethanol (5mg./100ml.) gave a smooth light-absorption curve devoid of maxima at wavelengths greater than 210mμ. Maxima did not appear when the ethanolic solution was made alkaline or acid, with enough potassium hydroxide or concentrated hydrochloric acid to give 0·1N solutions. The infrared spectrum of the hatching factor dissolved in chloroform (8mg./0·2ml.) showed bands at 2920 (s), 2850 (s), 1712 (s), 1610 (w), 1468 (w), 1379 (m), 1162 (m), 1038 (w) cm.$^{-1}$.

Each of the following tests was made with 1mg. of hatching factor, where applicable with procedures given by Cheronis (1954). The hatching factor reacted rapidly with 2% potassium permanganate solution, but caused little or no discoloration of bromine–water. It gave a positive iodic acid test. When mixed with a saturated solution of 2,4-dinitrophenylhydrazine in 2N-hydrochloric acid, a solution of the hatching factor became turbid after 5min., and slowly deposited a slight orange–yellow precipitate. Dimedone did not produce a precipitate. The factor reacted with 2,3,5-triphenyltetrazolium bromide, and Benedict's and Tollens's reagents, but not with Molisch's and Schiff's. It slightly changed the colour (from yellow to a light tan) of alcoholic ferric chloride (cf. Marrian *et al.* 1949). A chloroform solution of the hatching factor gave no coloration with tetranitromethane. It gave a positive iodoform test, and gave a red solution (light-pink on dilution) in a Zimmermann test (Corker, Norymberski & Thow, 1962). Tests for α- and β-unsaturated γ-lactones with sodium nitroprusside and 2,6-dichlorophenol-indophenol (Kuehl, Linstead & Orkin, 1950) were negative, as was the test with ceric nitrate reagent. Treatment with aqueous sodium nitrite, which gives a purple colour with α-unsubstituted tetronic acids (Haynes & Plimmer, 1960), gave a pale-yellow solution.

Paper chromatography. Solvent systems were limited to acid and neutral material because the hatching factor is unstable in alkali. Polar systems were needed for the hatching factor to run as a discrete spot. The R_F values of the hatching factor were obtained by plotting the R_F values of the strips (0·05 R_F unit in width) against the number of larvae hatched by the washings.

The following results (expressed as the cumulative larval hatch for 100 cysts over a 3-week period,

for successive strips from R_F 0·65 to 0·95) were obtained with solvent system (*c*): 48, 50, 142, 875, 721, 49. The hatch in the other strips did not differ significantly from that in water (i.e. 25 larvae/100 cysts). The R_F values obtained with the various solvent systems were, for maximum hatching activity: solvents (*a*) and (*c*), 0·80–0·85; solvents (*b*), (*d*), (*e*), (*f*) and (*g*), 0·85–0·90. Hatching activity travelled as a spot with large R_F values and with activity usually detectable over a range 0·15–0·20 R_F unit. Activity was recovered almost completely from the chromatograms. The active regions of the chromatograms showed a weak light-blue fluorescence in ultraviolet light; they also showed an orange spot after treatment with Brady's reagent, and with solvent systems (*b*) and (*d*)–(*g*) a brown spot after spraying with aniline–xylose reagent (Smith, 1958).

Solvent system (*f*) was used by Carroll *et al.* (1958) for the chromatography of the hatching factor, but they failed to find any active material on such chromatograms. Widdowson & Wiltshire (1958) reported R_F about 0·70 with solvent system (*d*), but with our conditions, using the same solvent system, the region of maximum hatching activity was at R_F 0·85–0·90. With this system additional solvent in the lower part of the chromatography chamber and working at 15°, or colder, helped to prevent distortion of the active spot.

Upward elution chromatography of the hatching factor from roots of tomato plants in solvent system (*b*) (Massey & Neal, 1953) gave hatching activity centred at R_F 0·84. In our experiments, using the hatching factor from potatoes, maximum hatching activity was in the region R_F 0·85–0·90 with the solvent system (*b*) and upward or downward development. Paper chromatograms developed with aqueous phenol had to be thoroughly air-dried, because traces of phenol inhibited larval hatch and caused irregular results. Water-saturated phenol was also a suitable eluent for developing chromatograms.

DISCUSSION

Among the procedures previously used to obtain crude preparations of the hatching factor from dilute aqueous solutions, only those mentioned by Calam, Raistrick & Todd (1949), Carroll *et al.* (1958), Widdowson & Wiltshire (1958) and Hartwell *et al.* (1960) have applied to large volumes of raw diffusate. For most of our preparations, the hatching factor was first concentrated by adsorbing it on charcoal. Although this removed nearly all the activity from the diffusate, the average recovery by elution of the charcoal was only 31%, and the proportion differed considerably at different times. An average recovery of 60% was reported by Widdowson & Wiltshire (1958) in 34 experiments with small volumes of diffusate. Calam *et al.* (1949), who also used charcoal adsorption, reported recoveries of 60–70% of the activity, but Carroll *et al.* (1958), using an improved bioassay technique, reported recoveries of 10% or less.

Asatoor & Dalgleish (1956) suggested the use of charcoal deactivated with stearic acid for the selective adsorption of aromatic compounds from aqueous solutions. This deactivated charcoal readily adsorbed the hatching factor present in potato-root diffusate, but less activity was recovered from phenol eluates than was obtained from untreated charcoal by elution with acetone–water (Widdowson & Wiltshire, 1958). Acetone or acetone–water mixtures, which eluted the hatching factor from deactivated charcoal, also eluted the adsorbed stearic acid.

The losses are greatest during the preparation of the crude extracts, and the great increase in activity for unit weight of product obtained from our further procedures leads to only small losses of activity. Extraction of active material from small volumes of aqueous solution with ethyl acetate proved much superior to the solvents (ether, butan-1-ol and phenol) previously used to extract the hatching factor because it combines satisfactory extracting properties with volatility. After its use column chromatography gave a colourless, very active, gum. The hatching factor is a readily oxidizable acid, probably with little carbon–carbon unsaturation, and containing the group $CH_3.CO$–. Other workers (Marrian *et al.* 1949; Widdowson & Wiltshire, 1958; Carroll *et al.* 1958; Hartwell *et al.* 1960) have obtained active materials by different methods, but there is too little information to assess the relative purity of these preparations. However, the product obtained by Widdowson & Wiltshire (1958) can be further purified by the methods reported here. Marrian *et al.* (1949), who used tomato-root leachings as their principal source of the hatching factor, fractionated the crude material obtained by adsorption on charcoal by unsatisfactory methods that largely or completely inactivated the material. Carroll *et al.* (1958) reported preliminary work on alternative procedures, but give no details. Some points of difference exist between our preparation from potato-root leachings and those obtained by Marrian *et al.* (1949) and Hartwell *et al.* (1960) from tomato roots. Marrian *et al.* (1949) reported that an ethanolic solution of their material showed a strong inflexion at 285 mμ, and gave a reddish-brown coloration with ferric chloride. Our preparation showed neither of these properties. Hartwell *et al.* (1960) reported the isolation of active crystalline material from tomato-root leachings. The hatching factor obtained from potato-root leachings by our pro-

cedures was too soluble in the solvent mixture (*tert.*-butyl alcohol, ethanol, ether) used by Hartwell *et al.* (1960), or in the individual components, to permit crystallization. The product after four crystallizations had little activity and at a concentration of 0·5 μg./ml. gave a hatch of fewer than two larvae/cyst. The responsiveness of *H. rostochiensis* cysts to the hatching factor differs from time to time, but a typical result with our purified product at a concentration of 0·05 μg./ml. was a hatch of 19 larvae/cyst during a period of 1 week; successive fourfold dilutions of this solution similarly hatched respectively 11, 7 and 4 larvae/cyst. Hence our product that failed to crystallize gave a similar hatch to that reported for the crystalline material when our product was 640 times as dilute.

Diffusate from tomato and potato roots give similar hatching curves (larval emergence/dilution) with *H. rostochiensis* cysts, and the differences reported between the hatching factors for the two plants probably indicate difference in degree of purity rather than of the structure of the active material.

The extensive bioassays and the production of the large volumes of root diffusates used in this work were done first under the supervision of E. Widdowson, and later of P. Nelson, M. Gander, P. Cox and A. Shepherd, to whom we gratefully acknowledge our indebtedness.

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Dealkylation Studies on Inhibited Acetylcholinesterase

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1. A rapid method for measuring the rate of dealkylation of organophosphorus-inhibited enzymes is described. 2. The dealkylation rates have been measured for acetylcholinesterase inhibited by isopropyl, *sec.*-butyl, 1,2-dimethylpropyl, 1,2,2-trimethylpropyl and cyclohexyl methylphosphonofluoridate, and by di-isopropyl phosphorofluoridate; they are first-order under the conditions used.

Cholinesterase inhibited by organophosphates can be reactivated by powerful nucleophiles such as hydroxamic acids and those based on pyridinium aldoximes, e.g. pyridine-2-aldoxime methiodide (Childs, Davies, Green & Rutland, 1955). Hobbiger (1955) observed that the extent of reactivation with nicotinohydroxamic acid as reactivator depended on the length of time the inhibitor had been in contact with the enzyme. The change in the inhibited enzyme to a form that cannot be reactivated by oximes has become known as 'aging', and has been observed with several enzymes (Berends, 1964). It has also been shown that the speed of 'aging' depends to a marked degree on the structure of the organophosphate used for inhibition, di-isopropyl phosphoryl-acetylcholinesterase 'aging' more rapidly than diethyl phosphonyl-acetylcholinesterase (Hobbiger, 1957).

Loomis & Salafsky (1963) showed that oximes were virtually ineffective in reactivating acetylcholinesterase inhibited by Soman (1,2,2-trimethylpropyl methylphosphonofluoridate) and that there was a 100% mortality in mice poisoned by Soman despite the administration of atropine and oxime. They suggested that rapid 'aging' could account for these observations.

Jansz, Brons & Warringa (1959) observed that the degradation of di-isopropyl phosphorofluoridate (DFP)-inhibited chymotrypsin, trypsin and liver ali-esterase gave serine residues that contained the di-isopropyl phosphoryl group, whereas pseudocholinesterase under the same conditions gave a monoisopropyl phosphorylserine derivative. It is to be noted that, for chymotrypsin, trypsin and liver ali-esterase, when this 'aging' phenomenon occurs at all it is considerably slower than for pseudocholinesterase. On this basis Jansz *et al.* (1959) suggested that 'aging' was caused by dealkylation of the dialkyl phosphoryl-enzyme. This hypothesis has been established for pseudocholinesterase by Berends, Posthumus, Sluys & Dieirkauf (1959), who have shown that the rate of 'aging' is the same as the rate of dealkylation. Usdin, Mitz & Killos (1964) report that other inhibitors behave similarly but no details have been given.

It was considered desirable to measure the rates of dealkylation of *sec.*-alkyl methylphosphonyl-acetylcholinesterases to assess the effect of the *sec.*-alkyl group on the rate of dealkylation, as certain *sec.*-alkyl groups increase the rate of 'aging'. The reactions involved in these measurements of dealkylation are given in Scheme 1.

MATERIALS AND METHODS

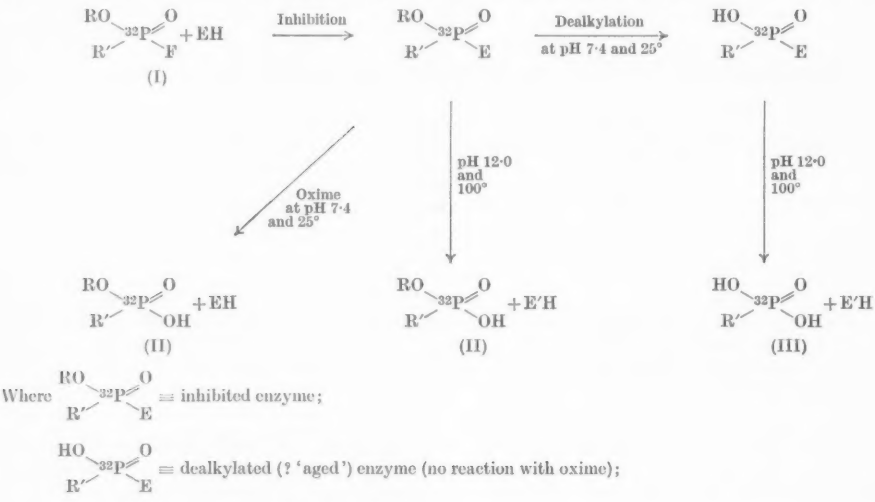
Enzyme. Aqueous dilutions of bovine erythrocyte acetylcholinesterase (Winthrop Laboratories Inc., New York, N.Y., U.S.A.) was used at concentrations of approx. 100 units/ml. The enzyme was found to contain no measurable amounts of ali-esterase or arylesterase.

Inhibitors. The ^{32}P-labelled inhibitors were prepared at Suffield Experimental Station, Ralston, Alberta, Canada (for their preparation see Reesor, Perry & Sherlock, 1960) and were shown to be at least 95% pure by the Schönnemann method (Marsh & Neale, 1956) with the exception of the di-isopropyl phosphorofluoridate which was 93% pure. The initial specific activities used were approx. 120 mc/g. The nature of the impurities is not known.

Radioactive counting. All the counts were made with a Geiger–Müller counter for liquids of the type CV2145 housed in a lead 'castle' and connected to an Ekco automatic scaler, type N530B. A 5 ml. volume of solution was used in each determination, and the count was over a period of 1000 sec. Correction for the background count was applied in each case.

Column chromatography. The following column-chromatographic procedures were used.

(1) Sephadex (Pharmacia, Uppsala, Sweden) was used to separate the protein containing the inhibited acetylcholinesterase from the excess of inhibitor and hydrolysis



Where ≡ inhibited enzyme;

 ≡ dealkylated (? 'aged') enzyme (no reaction with oxime);

EH ≡ enzyme; E′H ≡ denatured enzyme.

Scheme 1. Reaction products of *sec.*-alkyl methylphosphonofluoridates and cholinesterases.

products of the inhibitor. Sephadex G-50 (medium grade) in water was used to make up columns (40 cm. × 3 cm.). The protein was eluted with water, 5 ml. samples of the effluent were collected and the radioactivity of each was determined. Each column was only used once.

(2) Amberlite (British Drug Houses Ltd., Poole, Dorset) was used to separate the *sec.*-alkyl hydrogen methylphosphonate (Scheme 1, compound II) from the methylphosphonic acid (Scheme 1, compound III) when these acids had been produced as the alkaline-hydrolysis products of partially 'aged' inhibited acetylcholinesterase. Amberlite CG-120 type II resin, which had been washed by the continuous-flow technique to remove fine particles, was filtered, stirred with 2 M-LiCl–N-HCl and used to prepare the column. Sufficient resin to give a column 10 cm. high was poured in the form of a slurry into a 30 cm. × 2 cm. column surrounded by a water jacket maintained at 50° by a continuous-flow system. The base of the column was supported by a filter-paper disk bedded on acid-washed sand. Eluent solution (700 ml.) was passed down the column before use (flow rate approx. 0·4 cm./min.), and when the column had settled a disk of filter paper covered by a little acid-washed sand was used to preserve an even surface. Between runs, 300 ml. of eluent solution was passed down the column.

For isopropyl methylphosphonofluoridate (Scheme 1, compound I, R = isopropyl, R′ = methyl) and di-isopropyl phosphorofluoridate (Scheme 1, compound I, R = isopropyl, R′ = isopropoxy) a column of resin 20 cm. long was used and elution was effected with 4 M-LiCl–2 N-HCl.

Phosphorus analysis. An aqueous solution of the alkyl hydrogen methylphosphonate or methylphosphonic acid (4 ml.), containing an equivalent of up to 60 μg. of phosphorus, was decomposed by adding N-NaOH (1 ml.) and 10% (w/v) $(NH_4)_2S_2O_8$ (1 ml.) and heating at 100° for 30 min. After cooling, 5 N-H_2SO_4 (2 ml.) was added and the solution was diluted to 8 ml. and mixed. Then 1 ml. of 10% (w/v) sodium molybdate solution was added with stirring, followed by 1 ml. of 0·2% $SnCl_2$ solution and further mixing. After 5 min. the intensity of the blue colour formed was measured spectrophotometrically and the concentration of phosphorus read off from a calibration curve prepared from KH_2PO_4 solutions of suitable concentrations (Fiske & Subbarow, 1925).

Measurement of dealkylation. Each estimation required a 10 ml. sample of enzyme in 0·01 M-phosphate buffer, pH 7·4, and eight to ten samples were used for each determination of dealkylation rate. These were maintained at 25° with an additional sample that was used as a control (see below). Inhibition was carried out with a concentration of inhibitor that gave 100% inhibition in 1 min. This was 0·5 μM for the *sec.*-alkyl methylphosphonofluoridates and 1 μM for di-isopropyl phosphorofluoridate. Each solution was allowed to 'age' for an appropriate time-interval before the reaction was stopped by the addition of 6 g. of urea to the solution. The urea solution obtained was heated at 100° for 5 min. Acetylcholinesterase has been reported to be irreversibly denatured in 8 M-urea (Coleman, 1957), and since Berends (1964) has shown that the dealkylation process does not occur with denatured inhibited butyrylcholinesterase no further dealkylation was expected. However, preliminary studies showed that dealkylation was resumed on removal of urea from cyclohexyl methylphosphonyl-acetylcholinesterase by Sephadex separation. It appears that at least some of the functional groups at the active centre of the enzyme resume their original positions after the removal of

urea. The procedure described above was used since it was thought that dealkylation might be initially accelerated by heat treatment alone.

The denatured inhibited enzyme was placed on a Sephadex column and eluted with water. The radioactive protein fraction was found to separate completely from the excess of inhibitor and its hydrolysis products. The radioactive protein fraction was treated with N-NaOH to bring the pH of the solution to 12·0 and then heated at 100° for 1 hr. to liberate the *sec.*-alkyl hydrogen methylphosphonate and methylphosphonic acid. Chromatography on an Amberlite column established that there was no dealkylation of *sec.*-alkyl hydrogen methylphosphonates under these conditions.

The two expected phosphorus-containing acids were added to the hydrolysate to act as carriers (1 ml. of a 0·1 N solution of each acid). The solution was then reduced in volume to approx. 1 ml. and quantitatively transferred to the Amberlite column by using 6 ml. of the LiCl–HCl eluting solution. The column was developed and the effluent collected in 5 ml. fractions. Each fraction was examined for total radioactivity and total phosphorus content. In this manner it was established that the retention volumes for the radioactive phosphorus-containing acids corresponded to those of the carrier acids used. The order in which the acids were eluted from the column was determined beforehand and in every case the dealkylated phosphonic acid preceded the alkylated acid. This separation procedure was based on the work of Jakob, Park, Ciric & Rieman (1961), who have shown that under carefully controlled conditions a quantitative separation of phosphorus-containing esters and acids can be achieved with a Dowex cation-exchange resin of low capacity. The recovery of the phosphorus-containing acids from the Amberlite resin was 95–100%.

The possibility that non-specifically bound phosphorus was present in the deactivated enzyme was examined, since Cohen & Warringa (1953) have shown that non-specific phosphorylation can occur in some preparations of acetylcholinesterase. It was assumed that the binding of the phosphorus residue to sites other than the active site of the enzyme would be unaffected by denaturation. A control sample of the enzyme, which was taken for each inhibitor, was denatured with urea before treatment with the inhibitor. The sample was then subjected to the procedure outlined above and the amount of bound ^{32}P determined. In no case did the activity of the control sample exceed 5% of that of the other samples and no dealkylation of the non-specifically bound phosphorus was found to occur. Corrections for non-specifically bound phosphorus were made, based on the values determined in this way.

A further possible source of error lay in spontaneous dephosphonylation of the inhibited enzyme, equivalent to the spontaneous reactivation that has been observed in some organophosphorus-inhibited enzymes (Aldridge & Davison, 1953). The influence of this on the rate measurements would depend on whether the dephosphonylation of the dealkylated enzyme occurred more rapidly than the dephosphonylation of the alkylated enzyme. This possibility was studied by determining the radioactivity of the enzyme after its separation on the Sephadex column. Equivalent samples were examined after they had been standing for up to 72 hr. The radioactivity in the samples showed that the extent of dephosphonylation was no more than 5% after 72 hr. and was probably much less. It was not possible to correct for this in the rate values.

From the amount of the radioactive alkyl hydrogen methylphosphonate and the sum of the esterified and unesterified radioactive methylphosphonic acids from each sample the percentages of alkylated and dealkylated methylphosphonyl-acetylcholinesterase were obtained.

RESULTS AND DISCUSSION

When the logarithm of the percentage of alkylated methylphosphonyl-acetylcholinesterase present in each sample was plotted against the time of standing, a straight line was obtained, suggesting first-order kinetics. Fig. 1 shows such a plot for 1,2,2-trimethylpropyl methylphosphonyl-acetylcholinesterase, the most rapid of the dealkylations. In each case agreement with first-order kinetics was found when the results were subjected to a modification of the least-squares treatment described by Bond (1935), which takes the logarithmic nature of the concentration term into account. The inhibitors studied and the rates of dealkylation obtained are given in Table 1.

Di-isopropyl phosphorofluoridate has been included to compare the behaviour of acetylcholinesterase with that of pseudocholinesterase studied by Berends *et al.* (1959). Di-isopropyl phosphorofluoridate-inhibited acetylcholinesterase was found to dealkylate more slowly than the corresponding inhibited pseudocholinesterase. These

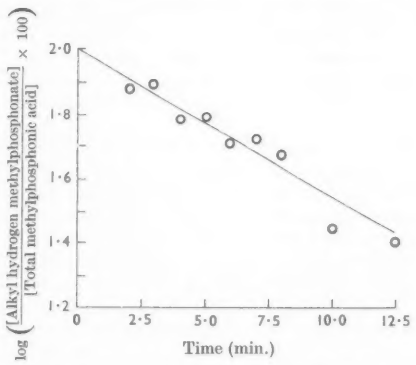
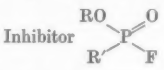


Fig. 1. Time-course of the spontaneous dealkylation of ^{32}P-labelled Soman-inhibited acetylcholinesterase at pH 7·4 and 25°. The dealkylation process in the samples was stopped by the addition of urea and heat treatment; after removal of excess of inhibitor by Sephadex separation, the inhibited enzyme was hydrolysed and the dealkylated and alkylated methylphosphonic acids formed were estimated by column chromatography and radioactive counts on the effluent.

Table 1. *First-order rate coefficients for the dealkylation of organophosphorus-inhibited acetylcholinesterase at* pH 7·4 *and* 25°

The rate coefficients were obtained from first-order kinetics applied to the percentage of alkyl methylphosphonyl-acetylcholinesterase present after standing for a known time at pH 7·4 and 25°. The method of estimating the percentage of alkyl methylphosphonyl-acetylcholinesterase is given in the text.

Inhibitor 

Trivial name	R	R′	10^3k (min.$^{-1}$)	Half-life (min.)
Sarin	Isopropyl	Methyl	0·37	1870
—	*sec.*-Butyl	Methyl	8·22	85
—	1,2-Dimethylpropyl	Methyl	12·0	58
Soman	1,2,2-Trimethylpropyl	Methyl	115	6
—	Cyclohexyl	Methyl	0·17	4000
DFP	Isopropyl	Isopropoxy	0·32	2140

workers used two methods to determine the rate of dealkylation of the inhibited enzyme. In the first, excess of ^{32}P-labelled di-isopropyl phosphorofluoridate was removed from the inhibited enzyme preparation by dialysis at low temperature before the enzyme was allowed to age at 24°. Samples were taken at intervals and subjected to alkaline hydrolysis. Radioautography of chromatograms of the hydrolysed samples showed the presence of radioactive di- and mono-isopropyl phosphates, which were estimated quantitatively. In the second method, di-isopropyl phosphorofluoridate labelled with ^{14}C in the isopropyl groups and with ^{32}P was used. It was shown that the ^{14}C content of the inhibited enzyme decreased at the same rate as the inhibited enzyme 'aged', whereas the ^{32}P content remained unchanged. The dealkylation step was found to have a half-life of about 5 hr. at 24°.

For our studies with *sec.*-alkyl methylphosphonofluoridates it was necessary to modify Berend's (1964) first method so that faster dealkylation rates could be measured. The method used is more rapid than that used by Berends *et al.* (1959) as Sephadex separation replaces dialysis and column chromatography, and liquid counting on the effluent replaces paper chromatography and radioautography of the chromatograms. The concentration values obtained by extrapolating the plots to zero time correspond to $100 \pm 4\%$ of fully alkylated phosphorus residue on the enzyme for all the inhibitors examined, and this is a measure of the accuracy of the method. However, the rates would be expected to vary with the environment of the enzyme and it would be dangerous to extend these results to other situations *in vitro* and the situation *in vivo* without experimental support. Extrapolation to 100% alkyl methylphosphonyl-acetylcholinesterase supports the suggestion that the urea and heat treatment of the inhibited enzyme stops the dealkylation process by denaturing the enzyme; no other means of denaturing the enzyme was tried in these studies.

It is clear that a wide variation in the dealkylation rate exists for the different phosphonylating groups under the conditions used. These differences in rate parallel the ease of formation of carbonium ion from the secondary alkyl group (Hughes, 1951). 'Aging' studies (D. B. Coult, D. J. Marsh & G. Read, unpublished work) carried out in connexion with this work show that 'aging' and dealkylation can only be considered synonymous under special circumstances.

Since this work was completed a study of the 'aging' and dealkylation of Soman-inhibited acetylcholinesterase has been published by Fleischer & Harris (1965). These workers used a different technique to determine the rate of dealkylation, for which they found a rate coefficient of 0·29 min.$^{-1}$ at 37° and pH 7·4, which is in reasonable agreement with the value of 0·115 min.$^{-1}$ at 25° and pH 7·4 found in the present study when the temperature difference is taken into account. They also found that the rate of 'aging' was not significantly different to that of dealkylation, which indicates that 'aging' and dealkylation are very closely connected.

These studies indicate that from a practical point of view any alkyl methylphosphonofluoridate in which the alkyl group easily forms a carbonium ion would form a rapidly dealkylating inhibited acetylcholinesterase, and poisoning by this compound would be resistant to therapeutic treatment with oximes and atropine.

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Activities of some Enzymes concerned with Citrate and Glucose Metabolism in Transplanted Rat Hepatomas

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1. Certain enzymes concerned with citrate and glucose metabolism have been measured in two transplanted rat hepatomas, one induced with ethionine (minimal deviation type) and one induced with dimethylaminoazobenzene. In these hepatomas both citrate-cleavage enzyme and NADP-linked isocitrate dehydrogenase in the soluble fraction of the cell were approximately one-third of the values for normal rat liver. These changes have been discussed in relation to the increased citric acid content of tumours and depressed rate of fatty acid synthesis. 2. The glucose–ATP-phosphotransferase activity was below normal liver values in the ethionine-induced tumour but greater than normal in the dimethylaminoazobenzene-induced hepatoma. The apparent K_m values for the glucose–ATP phosphotransferases of these hepatomas were approx. 8×10^{-5}M; no evidence was found for an enzyme with a high K_m for glucose equivalent to liver glucokinase. 3. Of the enzymes of the pentose phosphate pathway, glucose 6-phosphate-dehydrogenase activity was three to five times as great whereas 6-phosphogluconate-dehydrogenase activity was the same or lower than normal liver in the ethionine- and dimethylaminoazobenzene-induced tumours respectively.

Recently interest has been focused on the role of citrate-cleavage enzyme in fatty acid synthesis and on the key part played by citrate concentration in controlling carbohydrate and fat metabolism (Lowenstein, 1964; Srere, 1965). The activity of citrate-cleavage enzyme in tumours is of particular interest in view of the observations by Dickens (1941) that many tumours have a high citric acid content. Further, there is the general finding that tumours have a decreased ability to synthesize fatty acids (Medes, Friedmann & Weinhouse, 1956; Weber, Morris, Love & Ashmore, 1961; Weber, Henry, Wagle & Wagle, 1964) and to form acetoacetate (Dickens & Weil-Malherbe, 1943) and cholesterol (Gore & Popják, 1962), although in certain cases hepatomas appear to have retained some of these synthetic pathways (Begg & Trew, 1956; Siperstein & Fagan, 1964).

$$\text{Citrate} + \text{CoA} + \text{ATP} \rightarrow \text{acetyl-CoA} + \text{oxaloacetate} + \text{ADP} + \text{P}_i \qquad (1)$$

Citrate-cleavage enzyme catalysing reaction (1) could clearly play a part in both the supply of acetyl-CoA for these reactions and in controlling the citric acid concentration of the cell.

The tumours used in this present work were transplanted hepatomas derived from primary liver tumours induced with ethionine or dimethylaminoazobenzene. The ethionine tumour was 'minimal deviation' in some respects: thus it contained at least 50% of the normal liver glucose 6-phosphatase, whereas the latter had virtually none of this enzyme (E. Reid, personal communication). In view of this marked difference, measurements were also made of certain enzymes concerned with glucose 6-phosphate metabolism, namely glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and glucose–ATP phosphotransferase. [This general term, employed by Sharma, Sharma, Donnelly, Morris & Weinhouse (1965), is used here to indicate phosphorylation of glucose by ATP irrespective of the K_m value of the enzyme. Elsewhere the enzyme with the low K_m for glucose ($< 1 \times 10^{-4}$M) has been called hexokinase (EC 2.7.1.1) and that with the high K_m glucokinase (EC 2.7.1.2), in accordance with general nomenclature.] The two dehydrogenases of the pentose phosphate pathway are of interest not only in relation to glucose 6-phosphate metabolism but also because they are key enzymes in fatty acid synthesis since they are believed to provide much of the NADPH required in this process (Matthes, Abraham & Chaikoff, 1963; Katz, Rognstad & Kemp, 1965).

METHODS

Materials. Commercial reagents were used with the exception of 6-phosphogluconate dehydrogenase, which was a partially purified preparation from liver and used in the assay of glucose 6-phosphate-dehydrogenase activity as described by Glock & McLean (1953). Nicotinamide nucleotides, CoA and hexokinase were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany.

Tumours. Transplantable hepatomas initially induced with DAB* or ethionine were developed at the Chester Beatty Research Institute in the Laboratories of Dr L. A. Elson and Dr E. Reid respectively and were made available for this work through the kindness of Dr E. Reid. Studies on the biochemical properties of these tumours by Dr Reid showed that the ethionine tumour was probably of the minimal deviation type with 50% of the normal rat-liver content of glucose 6-phosphatase whereas the DAB-induced tumour had none of this enzyme. The tumours were transplanted subcutaneously into rats of the hooded Norway strain and were used about 3 weeks later. The ethionine tumours used in these experiments were the thirteenth and sixteenth transplant generation and the DAB-induced tumours the twenty-third generation. The tumours were removed and stored in ice for approx. 1 hr. before preparation of homogenates for enzyme determination.

Preparation of tissue extracts. Portions of normal liver and tumour were homogenized, with a Potter homogenizer, in 3 vol. of ice-cold medium containing 150 mM-KCl–5 mM-$MgCl_2$–5 mM-EDTA–10 mM-mercaptoethanol and adjusted to pH 7·4 with $KHCO_3$. This is similar to the extracting medium used by Sharma, Manjeshwar & Weinhouse (1963). This preparation was centrifuged at 100000*g* (average) for 45 min. The supernatant fraction was dialysed against the same extracting medium for 1 hr. in the cold to decrease the blank values in certain of the enzyme determinations. This preparation was used for the determination of all the enzymes.

The stability of the NADP-linked dehydrogenases in this extracting medium was tested after storage at −15° for 2 months; only very minor changes occurred during this time, in most cases less than 10% change in activity. The stability of liver and tumour extracts were the same. In the results presented here citrate-cleavage enzyme and glucose–ATP phosphotransferase were measured immediately after dialysis but in certain experiments NADP-linked isocitrate dehydrogenase, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were measured after storage for a short period at −15°. It is notable that the low-K_m enzyme phosphorylating glucose is stable to storage at −15°.

Citrate-cleavage enzyme. The activity of citrate-cleavage enzyme was estimated by the colorimetric procedure described by Kornacker & Lowenstein (1965). A unit of enzyme activity is defined as the amount of enzyme catalysing the formation of 1 μmole of acetyl-CoA/hr. at 37°.

Glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. The activities of glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate–NADP oxidoreductase; EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (6-phospho-D-gluconate–NADP oxidoreductase; EC 1.1.1.44) were measured by the method of Glock & McLean (1953) with the exception that the amount of NADP was increased by a factor 2. A unit of enzyme is defined as the amount of enzyme catalysing the formation of 1 μmole of NADPH/hr. at 25° based on the initial velocity.

Isocitrate dehydrogenase. The activity of NADP-linked isocitrate dehydrogenase in the soluble fraction of the cell [L-isocitrate–NADP oxidoreductase (decarboxylating), EC 1.1.1.42] was measured essentially according to the method of Ochoa (1955) except that the substrate concentration was increased by a factor 2. This increased the period of linearity of the reaction. A unit of enzyme is defined as the amount catalysing the formation of 1 μmole of NADPH/hr. at 25°, based on the initial velocity.

Glucose–ATP-phosphotransferase activity. The glucose-phosphorylating activities of the dialysed high-speed supernatant fractions from rat liver and hepatomas were measured essentially according to the method of Sharma *et al.* (1963) but with the following modifications. The amount of purified glucose 6-phosphate dehydrogenase was increased to 0·5 i.u. (the preparation used contained 140 i.u./mg. of protein; C. F. Boehringer und Soehne G.m.b.H) and excess of 6-phosphogluconate dehydrogenase [partially purified $(NH_4)_2SO_4$ preparation] was added. The glucose concentrations used were 0·5 mM and 100 mM for the low-K_m and high-K_m enzymes found in liver but a concentration of 10 mM was used with the hepatoma extracts since, as shown below, this has a glucose–ATP phosphotransferase with a higher K_m value than that of liver hexokinase. The reaction was started with 0·04 ml. of the dialysed high-speed supernatant fraction of liver or hepatoma.

In this assay glucose 6-phosphate is converted into 6-phosphogluconate by excess of purified glucose 6-phosphate dehydrogenase and the NADPH formed is measured spectrophotometrically. The liver supernatant fraction contains the two dehydrogenases of the pentose phosphate pathway so that the 6-phosphogluconate formed will be further metabolized, producing an additional amount of NADPH. This has been allowed for by some authors and various factors have been used to convert the NADPH produced into glucose 6-phosphate formed; for example Sharma *et al.* (1963) has used a factor 1·7 for this conversion.

In this present work a similar method of correction was used to that employed in the routine assay of glucose 6-phosphate dehydrogenase in liver extracts (Glock & McLean, 1953), that is by the addition of excess of 6-phosphogluconate dehydrogenase; each molecule of glucose 6-phosphate then forms two molecules of NADPH by the combined action of the two dehydrogenases.

The $(NH_4)_2SO_4$ preparation of 6-phosphogluconate dehydrogenase was similar to that described by Glock & McLean (1953); this preparation contained no glucokinase or hexokinase activity; 0·3 unit was added to the assay medium, a unit being defined as the amount of enzyme catalysing the formation of 1 μmole of NADPH/min. at 25°. This is an amount slightly less than the purified glucose 6-phosphate dehydrogenase added to the assay medium. In the presence of excess of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase the values of μmoles of NADPH produced were divided by 2 to convert into μmoles of glucose 6-phosphate formed. For convenience in comparison of enzyme activities in this present study a unit of glucose–ATP-phosphotransferase activity is defined as the amount of enzyme catalysing the formation of 1 μmole of glucose 6-phosphate/hr. at 25°.

* Abbreviation: DAB, dimethylaminoazobenzene.

In studies of K_m values of liver and tumours, $(NH_4)_2SO_4$ fractions (20–50% and 50–75% saturation) were prepared from the dialysed high-speed supernatant extracts. The precipitated proteins were collected by centrifugation and were then dissolved in the KCl–$MgCl_2$–EDTA–mercaptoethanol medium described above and dialysed against this same extracting medium. The reaction velocity was determined from the initial rate of reaction. In Figs. 1–3, $1/v$ is the reciprocal of the extinction change at 340mμ/min.

The rate of reduction of $NADP^+$ in this and the other NADP-linked enzyme reactions was measured with a Unicam SP. 800 double-beam recording spectrophotometer with a constant-temperature cell housing and scale-expansion accessory.

Protein. The protein content of the high-speed supernatant fractions was estimated essentially according to the method of Lowry, Rosebrough, Farr & Randall (1951).

RESULTS

The comparison of the activities of some enzymes concerned with citrate metabolism and glucose oxidation in normal rat liver, in a transplanted hepatoma induced with ethionine (minimal deviation type in some respects) and in a transplanted hepatoma induced with DAB are shown in Table 1.

The activity of the citrate-cleavage enzyme in the transplanted hepatomas is approx. 30% of the value for normal rat liver. This difference is seen when the results are expressed as activity/g. of tissue or on the basis of the protein content in the supernatant fraction after centrifugation at 100000*g* for 45min. Reid (1962) has examined the various methods of expressing results and comparing values from tumours with normal tissues and has concluded that the activity/g. of tissue forms a suitable basis. The values in Tables 1 and 2 are also given as units/mg. of protein since this is also a widely used basis of expression.

The NADP-linked isocitrate-dehydrogenase activity in the soluble fraction of the cell is also markedly lower in hepatomas when compared with normal liver. This present result is not necessarily in direct contradiction to the evidence showing undiminished rate of isocitrate-dehydrogenase activity in tumour (Wenner, Spirtes & Weinhouse, 1952), since in these latter experiments the whole tissue was used as an acetone-dried preparation whereas the present experiments report only the activity of this enzyme in the soluble fraction.

In contrast with these enzymes, glucose 6-phosphate-dehydrogenase activity increases some three- and five-fold in the hepatomas induced with ethionine and DAB respectively, whereas 6-phosphogluconate-dehydrogenase activity remains almost unchanged in the ethionine tumour but fell to approximately half the normal liver value in the DAB-induced tumour. The relative activity of glucose 6-phosphate dehydrogenase/6-phosphogluconate dehydrogenase changed from 1:2 in the control liver to 1:0·2 in the DAB-induced hepatoma.

Table 1. *Activities of citrate-cleavage enzyme and of some NADP-linked dehydrogenases in normal liver and transplanted rat hepatomas*

Normal animals of the same age and weight as the tumour-bearing rats were used. The results are expressed as units/g. of tissue and units/mg. of protein contained in the supernatant fraction after centrifugation at 100000*g* for 45min. The units are μmoles of product formed/hr., the product being acetyl-CoA (as acetohydroxamate) for citrate-cleavage enzyme and NADPH for the three dehydrogenases. The temperature was 25° for all enzymes except citrate-cleavage enzyme, which was measured at 37°. The temperature coefficient factor to convert this last-named enzyme activity into units at 25° is 0·42. The results are given as means ± S.E.M. The values for the two transplanted hepatomas induced with DAB are given individually. For details see the Methods section.

		Transplanted hepatomas	
	Liver	Ethionine-induced	DAB-induced
No. of animals ...	10	9	2
Units of enzyme/g. of tissue			
Citrate-cleavage enzyme	98 ± 13	36 ± 4	20, 26
NADP-linked isocitrate dehydrogenase	1110 ± 71	240 ± 21	340, 427
Glucose 6-phosphate dehydrogenase	105 ± 13	312 ± 12	482, 542
6-Phosphogluconate dehydrogenase	201 ± 5	160 ± 13	100, 106
Units of enzyme/mg. of protein			
Citrate-cleavage enzyme	1·05 ± 0·14	0·56 ± 0·05	0·34, 0·43
NADP-linked isocitrate dehydrogenase	11·8 ± 0·76	3·80 ± 0·44	5·75, 7·00
Glucose 6-phosphate dehydrogenase	1·13 ± 0·13	4·86 ± 0·20	8·16, 8·88
6-Phosphogluconate dehydrogenase	2·15 ± 0·06	2·46 ± 0·20	1·68, 1·74
Protein content (mg./g. of tissue) of high-speed supernatant fraction	94 ± 2·7	65 ± 2·6	59, 61

Table 2. *Activity of glucose–ATP phosphotransferases in normal liver and transplanted hepatomas*

Results are expressed as μmoles of glucose 6-phosphate formed/hr./g. of tissue or μmoles of glucose 6-phosphate formed/hr./mg. of protein contained in the supernatant fraction after centrifugation at 100000*g* for 45 min. The temperature was 25°. The enzymes were estimated by following the rate of formation of NADPH at 340 mμ in the presence of excess of purified glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase; this yields 2 μmoles of NADPH/μmole of glucose 6-phosphate formed, and therefore the extinction readings have been halved to correct the results to μmoles of glucose 6-phosphate formed. The total glucose-phosphorylating activity was determined with a final glucose concentration of 0·1 M. The low-K_m enzymes were estimated with 0·5 mM and 10 mM final glucose concentrations for liver and hepatomas respectively. The high-K_m glucokinase was calculated by difference. The results are given as means ± S.E.M. The values for the two transplanted hepatomas induced with DAB are given individually. For details see the Methods section.

		Transplanted rat hepatomas	
	Liver	Ethionine-induced	DAB-induced
No. of animals ...	9	9	2
μmoles of glucose 6-phosphate formed/g. of tissue/hr.			
Total activity	139 ± 9	63 ± 7	152, 172
Low-K_m (hexokinase)	14 ± 2	63 ± 7	152, 172
High-K_m (glucokinase)	125 ± 8	No activity	No activity
μmoles of glucose 6-phosphate formed/mg. of protein/hr.			
Total activity	1·50 ± 0·10	0·97 ± 0·11	2·6, 2·8
Low-K_m (hexokinase)	0·15 ± 0·02	0·97 ± 0·11	2·6, 2·8
High-K_m (glucokinase)	1·34 ± 0·10	No activity	No activity
Protein content of high-speed supernatant (mg./g. of tissue)	94 ± 2·7	65 ± 2·6	59, 61

These results show that there is not a generalized overall depression in enzymic activity in these transplanted hepatomas and comparison with other values for hepatomas in the literature suggests that the present values fit into a common pattern (see Reid, 1962; Weber *et al.* 1964).

The total rate of glucose phosphorylation, by enzymes in the soluble fraction of the cell in the presence of excess of glucose, is somewhat depressed in the ethionine-induced tumour but is greater than normal in the DAB-induced hepatoma (Table 2). Comparison was made of this activity in the high-speed supernatant fraction in all three tissues; the glucose 6-phosphatase activity of the ethionine tumour should not therefore interfere in this estimation. The total glucose-phosphorylating activity may be somewhat underestimated if part of the phosphorylating activity is associated with the particulate fraction of the cell as it is in brain (Crane & Sols, 1953) and ascites-tumour cells (McComb & Yushok, 1959). It was found in the present work that the substrate concentration which is used for hexokinase assay in normal liver (0·5 mM) did not fully saturate the enzyme in the tumour extracts. This led to a study of the K_m values of the enzymes phosphorylating glucose in normal and tumour tissues. The results of these experiments are summarized in Table 3 and Figs. 1, 2 and 3. The apparent K_m values of the dialysed soluble fractions were 1×10^{-5} M for liver and about six- to seven-fold higher for the tumour extracts. It appeared from these studies that there was only one enzyme phosphorylating glucose in the tumour extracts with a relatively low K_m; the enzyme equivalent to glucokinase with a high K_m was absent.

The liver and tumour extracts were fractionated with ammonium sulphate and the K_m values for the 20–50% saturated ammonium sulphate fraction measured in each case. This showed clearly that the K_m for liver and for the hepatomas (ethionine- and DAB-induced) were different (see Figs. 2 and 3): values of 1×10^{-5} M and 8×10^{-5} M were obtained respectively. The small differences between the K_m values for fresh extracts and ammonium sulphate fractions may be accounted for by removal of trace quantities of substrate.

The relative hexokinase/glucokinase activities in liver and the K_m values for the 20–50% saturated ammonium sulphate fraction are in good agreement with other values in the literature (see Table 3). The 20–50% saturated ammonium sulphate fraction of the tumour extracts contained most of the hexokinase activity although higher concentrations

Table 3. *Apparent K_m values for glucose–ATP-phosphotransferase activities of normal rat liver and hepatomas*

Tissue	Preparation	K_m	
(*A*) Present values			
Liver	20–50% satd. $(NH_4)_2SO_4$ (4)	1×10^{-5} M	
Ethionine-induced tumour	(*a*) Dialysed supernatant (4)	$5{\cdot}5\times10^{-5}$ M	
	(*b*) 20–50% satd. $(NH_4)_2SO_4$	8×10^{-5} M	
	(*c*) 50–75% satd. $(NH_4)_2SO_4$	8×10^{-5} M	
DAB-induced tumour	(*a*) Dialysed supernatant (2)	$7{\cdot}0\times10^{-5}$ M	
	(*b*) 20–50% satd. $(NH_4)_2SO_4$	8×10^{-5} M	
	(*c*) 50–75% satd. $(NH_4)_2SO_4$	No activity	
(*B*) Published values		Low-K_m (hexokinase)	High-K_m (glucokinase)
Liver	(*a*) Unfractionated supernatant		
	Lange & Kohn (1961)	4×10^{-5} M	—
	Walker (1963); Walker & Rao (1963)	$3{\cdot}8\times10^{-5}$ M	1×10^{-2} M
	DiPietro, Sharma & Weinhouse (1962)	—	$1\text{–}2\times10^{-2}$ M
	(*b*) 20–50% and 60–75% satd.† $(NH_4)_2SO_4$		
	Viñuela, Salas & Sols (1963)	1×10^{-5} M	1×10^{-2} M
	Abraham, Borrebaek & Chaikoff (1964*a*)	5×10^{-5} M	$0{\cdot}8\times10^{-2}$ M
	(*c*) DEAE-cellulose fractionation		
	González *et al.* (1964) Peak A	$0{\cdot}6\times10^{-5}$ M	$1{\cdot}8\pm0{\cdot}03\times10^{-2}$ M
	González *et al.* (1964) B	$4{\cdot}4\times10^{-5}$ M	
	González *et al.* (1964) C	$13{\cdot}0\times10^{-5}$ M	
Foetal rat liver	Walker & Rao (1963)	4×10^{-5} M	
Tumours	3′-Me-DAB and DAB cholangiocarcinomas		
	Shatton *et al.* (1962)	50×10^{-5} M	$0{\cdot}5\times10^{-2}$ M*
	Morris hepatoma 5123A and 7800		
	Elwood, Lin, Cristofalo, Weinhouse & Morris (1963)	10×10^{-5} M	

* High-K_m enzyme found in one tumour composed of mixed cells, hepatocarcinoma and cholangiocarcinoma.
† 20–50% satd. $(NH_4)_2SO_4$ fraction contains low-K_m hexokinase; 60–75% satd. $(NH_4)_2SO_4$ fraction contains high-K_m glucokinase.

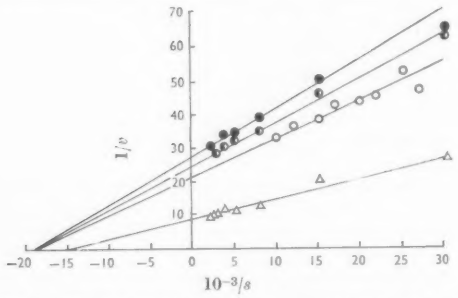

Fig. 1. Effect of variation in glucose concentration on the activity of glucose–ATP phosphotransferase of dialysed high-speed supernatant preparations (100000*g*) of the ethionine-induced tumours and DAB-induced tumour. These extracts were prepared as described in the Methods section. Three separate ethionine-induced tumours (○, ◐, ●) and one DAB-induced tumour (△) were used. The results are plotted according to Lineweaver & Burk (1934).

of ammonium sulphate precipitated additional hexokinase from extracts of the ethionine-induced tumour.

DISCUSSION

The central point of the present work is the activity of citrate-cleavage enzyme in tumours and the relationship of this to the overall pattern of carbohydrate and fat metabolism.

Citrate-cleavage enzyme

In transplantable hepatomas the activity of the enzyme was less than one-third of the value of normal rat liver. This might be expected to have two important consequences: first, to decrease the supply of acetyl-CoA for lipid synthesis, and secondly to increase the citrate concentration in the soluble fraction of the cell. There is indeed considerable evidence that hepatomas have a decreased ability to synthesize fatty acids (see Weber *et al.* 1964). The close parallelism between changes in citrate-cleavage enzyme and changes in the rate of fatty acid synthesis presented here has been noted in a variety of different conditions and tissues, e.g. in the livers of starving and re-fed animals (Kornacker & Lowenstein, 1963, 1965; Abraham, Kopelovich & Chaikoff, 1964*b*), in diabetic and insulin-treated rats (Kornacker &

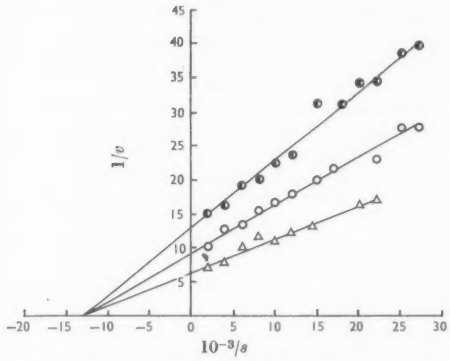

Fig. 2. Effect of variation in glucose concentration on the activity of glucose–ATP phosphotransferase of ethionine-induced tumour and DAB-induced tumour extracts fractionated with $(NH_4)_2SO_4$. The high-speed supernatant fractions from three ethionine-induced tumours were pooled and $(NH_4)_2SO_4$ fractions precipitating between 20–50% and 50–75% saturation were separated (○ and ◐ respectively). The DAB-induced tumour extract was treated similarly; activity was present only in the 20–50% saturated $(NH_4)_2SO_4$ fraction (△). The $(NH_4)_2SO_4$ fractions were prepared from extracts used in the experiments shown in Fig. 1. The results are plotted according to Lineweaver & Burk (1934).

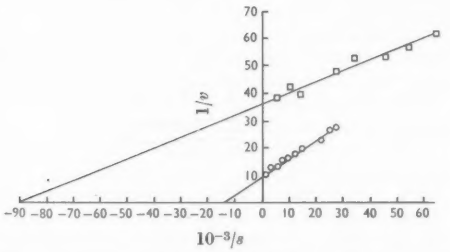

Fig. 3. Comparison of the effect of variation in glucose concentration on the activity of the low-K_m enzyme phosphorylating glucose in liver and ethionine-induced tumours. The fraction of the high-speed supernatant preparation that precipitated between 20 and 50% $(NH_4)_2SO_4$ saturation was used: □, liver; ○, ethionine-induced tumour.

Lowenstein, 1964), in hypophysectomized rats (Abraham *et al.* 1964*b*), in the mammary gland at different stages of lactation (Spencer, Corman & Lowenstein, 1964) and in adipose tissue of normal and diabetic rats (Brown & McLean, 1965). The present results suggest that this may also be true for hepatomas.

Dickens (1941) demonstrated that tumours have, in general, a high content of citric acid and that the citric acid concentration is greater in the rapidly growing peripheral portion of the tumour than in the central region. A decrease in the citrate-cleavage-enzyme activity could be of importance in raising the citrate content of the cell but this represents only one facet of the problem of control of the citrate concentration. The activities of the mitochondrial enzymes must also be considered. The available evidence indicates that both citrate synthetase and isocitrate dehydrogenase are present in tumours in amounts comparable with the activities in normal tissues, whereas, in contrast, aconitase activity is lower than in normal tissues (Wenner *et al.* 1952). Therefore the overall enzyme pattern in the two cell compartments in tumours could well lead to an increase in the citrate concentration, the key enzyme change being the fall in citrate-cleavage enzyme.

There is recent evidence for an additional extramitochondrial pathway of citrate formation from α-oxoglutarate or glutamate (D'Adamo & Haft, 1962, 1965; Madsen, Abraham & Chaikoff, 1964; Lardy, Shrago, Young & Paetkan, 1964; Srere, 1965). The results of isotopic experiments indicate the conversion of glutamate into citrate with subsequent enzymic cleavage of the citrate. The enzymes aconitase and NADP-linked isocitrate dehydrogenase are present in the soluble fraction of the cell where this pathway operates in the reverse direction to that normally occurring in the tricarboxylic acid cycle (see Srere, 1965). Part of this route of citrate formation has been measured in transplanted hepatomas in the present experiments. The observation that the soluble NADP-linked isocitrate dehydrogenase is low in the hepatomas compared with liver suggests that this route plays a more minor role in this tissue. It is noteworthy, in relation to the citrate concentration, that the relative activities of citrate-cleavage enzyme and NADP-linked isocitrate dehydrogenase remain favourable to citrate formation (Table 1).

An important implication of the accumulation of citrate in the cell is that citrate is an inhibitor of phosphofructokinase and could thus have a powerful effect on the control of the glycolytic pathway at this rate-limiting step. There is considerable evidence that metabolic control can be exerted at this stage in many tissues both by citrate accumulation (Parmeggiani & Bowman, 1963; Passonneau & Lowry, 1964) and by ATP and inorganic phosphate accumulation (Park *et al.* 1961; Newsholme & Randle, 1961; Passonneau & Lowry, 1964; Wu, 1964). There is evidence that the phosphofructokinase of Novikoff hepatomas is inhibited under aerobic conditions consistent with an inhibition by ATP (Wu, 1964). The extent of control by citrate

in tumours having a high glycolytic rate would be of interest, particularly since certain feedback control mechanisms are lost in tumours. An example in lipid metabolism is the loss in hepatomas of the cholesterol feedback mechanism at the point of conversion of β-hydroxy-β-methylglutaric acid into mevalonic acid (Siperstein & Fagan, 1964).

Glucose phosphorylation

There is now considerable evidence that glucokinase activity is greatly decreased in or absent from many hepatomas (Shatton, Donnelly & Weinhouse, 1962; Sharma *et al.* 1965). These authors have shown that in pre-cancerous liver there is a progressive rise in hexokinase to a value some five or six times as high as normal and a fivefold lowering in glucokinase. In a series of ten transplanted tumours eight had low or negligible glucokinase activity. The present results, with two types of transplanted hepatomas, fit the general pattern described by these authors. The changes in K_m of the glucose–ATP phosphotransferase involve not only the loss of the high-K_m glucokinase but also a change in K_m of the enzyme with glucose at low concentrations to a value some five to eight times the average for normal rat-liver hexokinase (Fig. 3).

The work of González, Ureta, Sánchez & Niemeyer (1964) showing multiple molecular forms of glucose–ATP phosphotransferase is of interest here since these authors were able to distinguish three isoenzymes with low K_m values. It is possible that the proportions of these isoenzymes are altered in hepatomas and studies with fractionation of these tumour extracts on DEAE-cellulose similar to those of Gonzalez *et al.* (1964) would be of value here. There is evidence for alterations in the lactate-dehydrogenase isoenzyme pattern in a variety of human tumours compared with adjacent normal tissue (Kaplan & Goodfriend, 1964) and Weber *et al.* (1964) have also shown that the K_m of phosphoglucomutase is higher in hepatomas than in normal liver. Similar alterations in enzymes controlling glucose phosphorylation may occur.

Pentose phosphate pathway

Weber *et al.* (1964) have examined a number of biochemical parameters in a range of transplanted hepatomas of different growth rates and have found that, in general, glucose 6-phosphate-dehydrogenase activity is increased. There is an increase in the activity of glucose 6-phosphate dehydrogenase as early as 2–4 weeks after feeding with a diet containing carcinogenic azo-dyes (Nodes & Reid, 1964; McLean, Reid & Gurney, 1964). The two transplanted tumours studied here followed this general pattern with a three- to five-fold increase in glucose 6-phosphate-dehydrogenase activity. By contrast, 6-phosphogluconate-dehydrogenase activity either remained unchanged or fell in the ethionine- and DAB-induced tumours respectively.

The very high glucose 6-phosphate-dehydrogenase activity and the marked alternations in the relative activities of the two dehydrogenases of the pentose phosphate pathway do not appear to fit readily the overall pattern of metabolism of the hepatomas. First, there is, in general, a decline in the total NADP content of most tumours and hepatomas, often to extremely low concentrations (Glock & McLean, 1957; Nodes & Reid, 1964; Greenbaum, Clark & McLean, 1965). Further, the reductive synthetic utilization of NADP for fatty acid synthesis is decreased and the dehydrogenases of the pentose phosphate pathway are thought to be tightly geared to this process (see Dickens, 1959). Thus the functional significance of this increased glucose 6-phosphate-dehydrogenase activity remains in question.

Many authors have speculated on the nature of the stimulus causing increased formation of glucose 6-phosphate dehydrogenase in such conditions as re-feeding after starvation. Glucose 6-phosphate, in particular, has been considered as a possible inducer, but no firm conclusion has been reached about this particular role of glucose 6-phosphate (Steiner & Williams, 1959; Potter & Ono, 1961; Bottomley, Pitot, Potter & Morris, 1963; Tepperman & Tepperman, 1963; Pitot, Peraino, Pries & Kennan, 1964). It seemed possible that the high glucose 6-phosphate-dehydrogenase activity of hepatomas might be related to an increased rate of formation of glucose 6-phosphate and some evidence for this is summarized in Table 4.

The glucose 6-phosphate of liver undergoes marked variation according to dietary conditions (Steiner & Williams, 1959). The alterations in the concentration of glucose 6-phosphate can be largely accounted for by the presence of the adaptive enzyme glucokinase. Time-sequence studies also show that glucokinase increases in activity before glucose 6-phosphate dehydrogenase (Potter & Ono, 1961; Pitot *et al.* 1964). Many hepatomas appear to lose the adaptable glucokinase activity found in liver and to have a high concentration of glucose–ATP phosphotransferase with a high affinity for glucose, conditions that would permit a rapid and constant rate of formation of glucose 6-phosphate. Another factor which could contribute to the increased availability of glucose 6-phosphate is the relatively low activity of glucose 6-phosphatase in these hepatomas (E. Reid, personal communication). Walker, Parry & Vernon (1965) have suggested that the activity of glucose 6-phosphatase rather than glucokinase might be the principal factor controlling the liver glucose 6-phosphate

Table 4. *Factors concerned in metabolism of glucose 6-phosphate in normal liver and hepatomas*

References: 1, Tepperman & Tepperman (1963); 2, Potter & Ono (1961); 3, Bottomley *et al.* (1963); 4, Pitot *et al.* (1964); 5, Shatton *et al.* (1962); 6, Sharma *et al.* (1965).

	Normal liver	Hepatoma	Reference
Glucose 6-phosphate dehydrogenase	100%	Increased up to 500%	1, 2, 3, 4
Glucose phosphorylation			
Hexokinase (low K_m)	Low	High	5, 6
Glucokinase (high K_m)	High	Low or absent	6
Re-feeding after starvation	Increased glucokinase followed by increased glucose 6-phosphate dehydrogenase	No change in activity	2, 3
Availability of glucose as substrate	Variable via portal circulation	Relatively constant in transplanted hepatomas via peripheral circulation	

concentration. There appears to be a relationship between conditions giving a high rate of formation of glucose 6-phosphate and a high glucose 6-phosphate-dehydrogenase activity.

It is thus postulated that glucose 6-phosphate may, in fact, be a regulatory factor for glucose 6-phosphate dehydrogenase.

Measurements of the glucose 6-phosphate content of liver and tumours are only partially in agreement with the role of glucose 6-phosphate in controlling glucose 6-phosphate dehydrogenase. Tumours, in general, appear to contain a very similar concentration of glucose 6-phosphate to that found in normal tissues (LePage, 1948). It is possible that the rate of formation of glucose 6-phosphate is the more critical factor, particularly since a glucose 6-phosphate-induced increase in glucose 6-phosphate-dehydrogenase activity would tend to maintain the concentration of the substrate at the normal value. Product inhibition of hexokinase by glucose 6-phosphate could also be of importance (Weil-Malherbe & Bone, 1951). The experiments of Steiner & Williams (1959) and of Tepperman & Tepperman (1963) are of particular importance here. The first authors, by measuring glucose 6-phosphate concentrations in the liver within a short time (20 min.) after administration of glucose, were able to show rapid changes which were not apparent when longer time-intervals were used.

There is evidence that glucose 6-phosphate dehydrogenase may be under various forms of metabolic control. One example is the inhibition of this enzyme by long-chain acyl-CoA derivatives (Eger-Neufeldt, Teinzer, Weiss & Wieland, 1965). Here, again, there is an interplay of factors between carbohydrate and fat metabolism; the decreased fatty acid synthesis of the tumour, perhaps in part mediated by changes in the citrate-cleavage enzyme, may release glucose 6-phosphate dehydrogenase from this inhibition.

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Changes in Dry Weight, Protein, Deoxyribonucleic Acid, Ribonucleic Acid and Reserve and Structural Carbohydrate during the Aerobic Growth Cycle of Yeast

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1. Changes in dry weight, DNA, RNA, protein and reserve and structural carbohydrate were measured during the aerobic growth of yeast on 0·9% glucose in an aerobic synthetic medium. 2. After glucose had been consumed and during the growth of yeast on ethanol and acetate, the rate of formation of DNA remained about the same but the rate of increase of dry weight was greatly diminished. 3. During the second stage of growth the ratios dry weight/DNA, protein/DNA, RNA/DNA and carbohydrate/DNA decreased to about 30% of the corresponding values during the first stage of growth. 4. A higher fraction of the dry weight of the yeast cells could be accounted for by the reserve carbohydrate content of the cells during the second stage of growth. 5. By the end of the first stage of growth an increase in the reserve carbohydrate content of the cells was observed. Part of this reserve carbohydrate was consumed by the cells in the beginning of the second stage of growth. The possibility of adaptation of cells at the expense of their reserves is discussed.

The metabolism of carbohydrate, protein and nucleic acids in yeast cells has been studied by many workers.

Thus in actively growing yeast the synthesis of polysaccharides was studied by Gilvarg (1952), Chung & Nickerson (1954) and Chen (1959*a*,*b*). In non-proliferating yeast the synthesis and degradation of carbohydrate in the presence of an exogenous source of carbon under anaerobic and aerobic conditions were examined by Fales & Baumberger (1948) and Fales (1951), and Fales (1960) described the effect of different degrees of aeration on the carbohydrate and fat content of the yeast. The endogenous metabolism of the reserve carbohydrate of anaerobically grown yeast under both aerobic and anaerobic conditions was examined by Chester (1963, 1964).

Protein and RNA turnover in growing and resting yeast cells was studied by Halvorson (1958*a*,*b*), and Shortman & Fukuhara (1963) failed to demonstrate any change in the rate of RNA synthesis during the respiratory adaptation of non-proliferating yeast cells.

The role of RNA in the growth of bacteria and the changes in the rate of RNA synthesis accompanying the changes in the growth rate were studied in detail by Schaechter, Maaløe & Kjeldgaard (1958), Kjeldgaard, Maaløe & Schaechter (1958) and Neidhardt & Magasanik (1960).

In the present paper changes in DNA, dry weight, RNA, protein and carbohydrate contents during the aerobic growth of yeast are examined. The aim of the work is to determine any major changes in the anabolic processes concomitant with the changes of structure and enzyme activity (Polakis, Bartley & Meek, 1964, 1965; Polakis & Bartley, 1965).

METHODS

Maintenance and growth of yeast. *Saccharomyces cerevisiae* strain no. 77 of the National Collection of Yeast Cultures (Brewing Industry Research Foundation, Nutfield, Surrey) was used. It was maintained on agar slopes of the synthetic medium for growth described by Polakis & Bartley (1965). Cells were grown at 35° on 0·9% glucose in the liquid synthetic medium described by the same authors. These are referred to below as the standard conditions of growth.

Determination of dry weight. Cells were washed several times at 0° and dried overnight at 105°.

Determination of glucose in the growth medium. Glucose was determined by the glucose-oxidase method of Huggett & Nixon (1957).

Determination of cell constituents. At the times indicated, four or five samples were removed from the growth medium. The cells were spun down and washed twice with ice-cold water at 0°. The samples were used for the determination of protein, dry weight, carbohydrate and nucleic acids.

Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). Crystalline bovine plasma albumin (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex) was used as standard.

Table 1. *Changes in dry weight, DNA, RNA, protein and carbohydrate contents of yeast cells during aerobic growth in synthetic medium on 0·9% glucose (50 μmoles/ml.)*

Methods of fractionation and determination are given in the Methods section. Reserve carbohydrate, trehalose+glycogen; structural carbohydrate, mannan+glucan.

Time of growth (hr.)	Glucose left in medium (μmoles/ml.)	Content (μg. in yeast cells present in 1 ml. of medium)											Recovery of total carbohydrate (%)
		DNA	RNA	Protein	Dry wt.	Trehalose	Glycogen	Reserve carbohydrate	Mannan	Glucan	Structural carbohydrate	Total carbohydrate: Calc.	Total carbohydrate: Determined	
14	42·7	0·23	19·2	108·8	328	1·8	15·6	17·4	21·4	16	37·4	54·8	—	—
16	21·3	0·41	39·1	230·0	620	2·6	43·3	45·9	35·3	32·8	68·1	114·0	—	—
18	0·04	0·56	54·8	320·0	882	17·8	97·5	115·3	51·9	49·2	101·1	216·4	—	—
19½	0·02	0·66	67·2	352·0	976	28·5	62·4	90·9	78·0	58·8	136·8	227·7	—	—
27	—	2·75	85·0	412·0	1550	66·1	68·6	134·7	108·2	67·7	175·9	310·6	327·9	94·7
28	—	3·03	92·2	462·0	1660	88·5	75·9	164·4	122·9	72·2	195·1	359·5	374·8	95·9
30	—	3·99	100·0	480·4	1880	99·4	97·1	196·5	149·4	100·3	249·7	446·2	466·1	95·7
36	—	5·08	109·2	545·0	2200	63·1	113·8	176·9	153·3	102·9	256·2	433·1	—	—
40	—	5·20	108·2	546·0	2240	58·4	136·9	195·3	150·2	114·6	264·8	460·1	—	—

Nucleic acids were fractionated by the method of Schmidt & Thannhauser (1945) as described by Hutchison & Munro (1961). DNA was estimated by the diphenylamine method of Burton (1956), all the quantities being scaled down to one-fourth. RNA was measured by the orcinol method, as described by Schneider (1957). Salmon-sperm DNA and yeast RNA were used as standards.

The reserve and structural carbohydrate was fractionated by the method of Trevelyan & Harrison (1956). The sum of acid-soluble glycogen and alkali-soluble glycogen is referred to as glycogen. Total carbohydrate and the amount of sugar in the different fractions were determined by the anthrone method, as described by Ashwell (1957). Samples and the anthrone reagent were chilled in an ice–water bath and mixed by vigorous shaking while immersed in the bath. The tubes were heated at 90° for 16 min. and then cooled, and the resulting green-coloured solutions were read at 625 mμ. Glucose and mannose were used as standards.

RESULTS

Yeast was grown aerobically in the synthetic medium containing glucose (0·9%) at 35°. At the times indicated, samples of cells were removed for determination of dry weight, DNA, RNA, protein and carbohydrate. The results of the experiments are given in Table 1.

Changes in DNA and dry weight. The changes in the amounts of DNA and dry weight of yeast cells in 1 ml. of growth medium during aerobic growth of the yeast are given in Fig. 1. In Fig. 1 the amounts of glucose left in the medium at different times are also presented. When glucose had been consumed there was an immediate fall in the rate of increase of

Fig. 1. Changes in DNA and dry weight of yeast cells during growth under standard conditions. ○, dry weight (mg./l.); □ DNA (μg./l.). The glucose remaining in the medium is also shown (△).

dry weight, but the rate of DNA synthesis was not affected. This is clearly shown in Fig. 2, in which changes in dry weight are plotted against the changes in DNA. At the time glucose disappeared from the medium, the rate of increase of dry weight changed from about 1500 μg. dry weight/μg. of DNA to about 300 μg. dry weight/μg. of DNA.

Changes in protein. Relative changes in the ratios protein/DNA and protein/dry weight are shown in Fig. 3. The values for protein/DNA and protein/dry weight at any time are expressed relative to the corresponding values after 14 hr. of growth, these being taken as unity. Initially, as cells were approaching the end of the growth period on glucose, there was a small increase in both ratios which during the second stage of growth fell below unity. Both increase and decrease were more pronounced for the protein/DNA ratio.

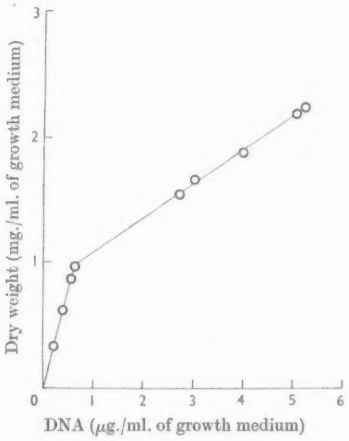

Fig. 2. Changes in the dry weight of yeast cells in relation to the change of DNA during growth under standard conditions. When glucose was consumed the slope of the line changed.

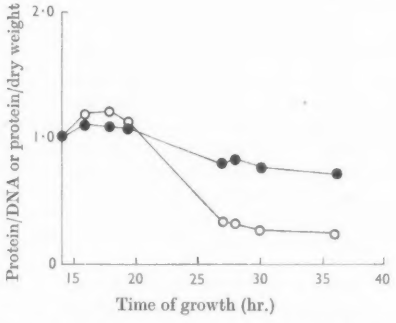

Fig. 3. Relative changes in the ratios protein/DNA and protein/dry weight during growth under standard conditions. The ratios are expressed relative to those after 14 hr. of growth, which were taken as unity. ○, Protein/DNA: ●, protein/dry weight.

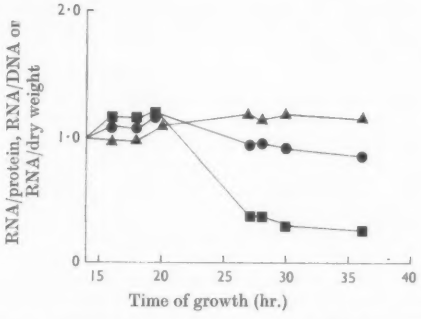

Fig. 4. Relative changes in the ratios RNA/protein, RNA/DNA and RNA/dry weight during growth under standard conditions. The ratios are expressed relative to those after 14 hr. of growth, which were taken as unity. ▲, RNA/protein; ●, RNA/dry weight; ■, RNA/DNA.

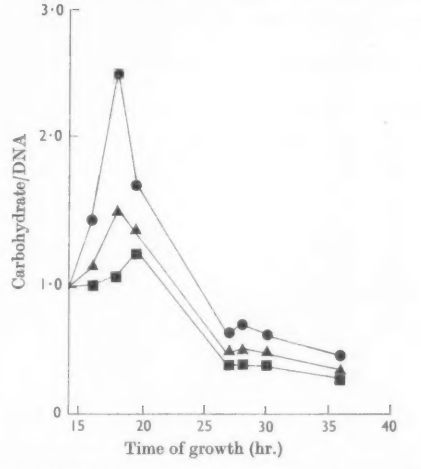

Fig. 5. Relative changes in the ratios carbohydrate/DNA during growth under standard conditions. The ratios are expressed relative to those after 14 hr. of growth, which were taken as unity. ●, Reserve carbohydrate/DNA; ■, structural carbohydrate/DNA; ▲, total carbohydrate/DNA.

Changes in RNA. Changes in the relative ratios RNA/DNA, RNA/dry weight and RNA/protein are presented in Fig. 4. Changes in RNA/DNA and RNA/dry weight followed the same pattern as the corresponding protein ratios. The RNA/protein ratio, which remained constant during growth on glucose, changed to another almost constant value 15% higher than the first one during the subsequent growth on ethanol and acetate, in spite of the decrease in the rate of growth of yeast (cf. Schaechter *et al.* 1958; Kjeldgaard *et al.* 1958; Neidhardt & Magasanik, 1960; Kjeldgaard, 1961; Neidhardt & Fraenkel, 1961).

Changes in carbohydrate. Changes in carbohydrate are shown in Figs. 5 and 6.

Initially there was a slow increase in the structural carbohydrate/DNA ratio (Fig. 5). In the second stage of growth the relative ratio fell to 0·3–0·4 (Fig. 5). The changes in structural carbohydrate/dry weight are presented in Fig. 6. The amount of structural carbohydrate varied very little in relation to dry weight. Reserve carbohydrate was the cell constituent subjected to the greatest changes during the growth cycle. Particularly noticeable was the increase in the amounts of reserve carbohydrate at the end of growth on glucose (Figs. 5 and 6). Both the relative ratios reserve carbohydrate/DNA and reserve carbohydrate/dry weight increased 2·5–3-fold. A part of this newly synthesized carbohydrate at the last stages of growth on glucose (16–18 hr.) was consumed during the subsequent 1·5 hr. of growth (18–19·5 hr.) (Table 1). The ratio reserve carbohydrate/DNA fell initially sharply in the beginning of the second stage of growth and then continued decreasing very slowly. In spite of this, during the growth of yeast on acetate and ethanol a higher fraction of the dry weight was composed of carbohydrate than during growth on glucose (Fig. 6). Of the two major components of carbohydrate, trehalose showed the greatest increase on passing from the first to the second stage of growth.

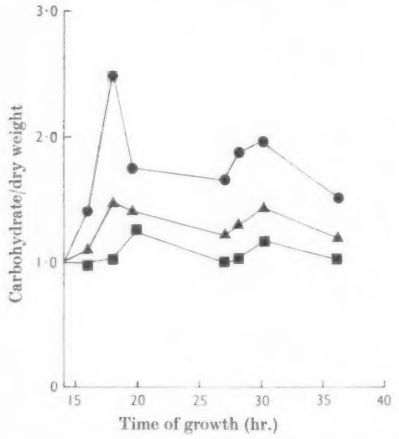

Fig. 6. Relative changes in the ratios carbohydrate/dry weight during growth under standard conditions. The ratios are expressed relative to those after 14 hr. of growth, which were taken as unity. ●, Reserve carbohydrate/dry weight; ■, structural carbohydrate/dry weight; ▲, total carbohydrate/dry weight.

DISCUSSION

Williamson & Scopes (1961) separated yeast into two fractions of different mean size and showed that, in spite of the wide differences in the mean content of large and small cells in RNA, protein and dry weight, the DNA content/cell was the same. If the DNA content/yeast cell is considered constant during growth of the yeast, then the results described in this section are consistent with a sharp decrease in the mean size of cells when glucose was consumed and growth continued on the degradation products of glucose. Electron microscopy confirmed the change in size. Dependency of cell mass on growth rate had also been observed by Schaechter *et al.* (1958). The bigger size of cells during growth at a higher rate in comparison with cells growing at a lower rate was ascribed by these authors to the need of an increase in the number of centres of protein synthesis (ribosomes) in the rapidly growing cells. The change in the size of the cells observed in this work resulted in an increase of the surface area for a given volume, thus possibly facilitating the uptake of different nutrients by the cells, including oxygen, as well as the excretion of the end products of metabolism.

The rate of increase in dry weight and DNA with time (Fig. 1) closely resembles the changes of the same constituents observed by Tustanoff & Bartley (1964) during anaerobic growth of yeast on glucose. The presence of glucose in the medium resulted in a high growth rate and the formation of giant cells, thus possibly decreasing the uptake of nutrients per given volume or mass of cells (assuming the density of cells to be constant). Any relation between this phenomenon and the repression by glucose of the respiratory and tricarboxylic acid-cycle enzymes cannot be assessed at present.

The increase in the carbohydrate content of the cells by the end of the first stage of growth could be attributed to an increased degree of aerobiosis as the glucose concentration in the medium was decreased to a low level. Fales (1960) studied the effect of the degree of aerobiosis on the carbohydrate content of yeast and showed that strong aeration

resulted in an increase in the synthesis of cellular carbohydrate. Brandt (1941) observed a parallelism between the trehalose content of yeast cells and the degree of aerobiosis, and S. L. Chen & K. J. H. Lauer (quoted in Chen, 1959*b*) also observed great increases in trehalose content on changing from anaerobic to aerobic conditions. Considering the fact that the presence of glucose in the growth medium results in a semi-anaerobic pattern of metabolism by the yeast cells, the results described in the present paper are in agreement with the work of the authors mentioned.

The sudden breakdown of these reserves in the beginning of the second stage of growth could suggest that yeast cells adapt to a fully aerobic type of metabolism at the expense of their reserve carbohydrate. In fact, Chester (1963) showed synthesis of cytochrome *c* oxidase during the aerobic dissimilation of reserve carbohydrate of anaerobically grown yeast and inhibition of dissimilation by inhibitors of protein synthesis.

The increased carbohydrate/dry weight ratio in the second stage of growth was possibly due to the development of the enzymes of the glyoxylate pathway supplying oxaloacetate, and to a preferential allotment of energy to the formation of reserve carbohydrate rather than protein and lipids (Polakis & Bartley, 1965, 1966).

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Metabolic Properties of Histones from Rat Liver and Thymus Gland

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1. The incorporation of ^{14}C-labelled amino acids into acid-extractable proteins from rat-liver and -thymus nuclei confirmed the existence of a protein component with a higher uptake than that into the major histone components. 2. This rapidly labelled component appeared to contain the thiol groups detectable in the acid extracts. 3. Histone f1 contained 1 mol. of serine phosphate/mol. of mol.wt. of 42000–43000. 4. Phosphate was present in other components of the 50mM-hydrochloric acid extract from liver and thymus nuclei, and was probably associated with the thiol-containing component. 5. The difference in amino acid uptake into the histones of diffuse and dense chromatin was confirmed. Dense chromatin was found to have a higher proportion of disulphide than did diffuse chromatin.

Examination of the histones that could be separated by preparative electrophoresis on polyacrylamide gel showed that when rat-thymus nuclei were incubated with ^{14}C-labelled amino acids there was a marked difference in rate of uptake of the amino acids into the different proteins, one component (6) having an especially high uptake (Ord, Raaf, Smit & Stocken, 1965). In the electrophoretic procedure used, 2–3 days were required for the separation of the different components, so that proteolysis could have occurred, and only 5mg. of material could be examined. To confirm the existence of a basic protein into which amino acids were incorporated rapidly, and to obtain sufficient material for the nature of the component to be investigated, it was decided to use other methods for the separation of the histones and also to see whether the component was found in histones from nuclei of another tissue. A comparison was also made of the pattern of amino acid and [^{32}P]-phosphate uptake into thymus histones *in vivo* and *in vitro*.

METHODS

Preparation of nuclei. Nuclei were isolated from liver and thymus glands of rats (120g. body wt.) in 0·25M-sucrose–5mM-$MgCl_2$–5mM-tris–HCl, pH 7·2, as described by Ord *et al.* (1965). The final concentration of the nuclear suspension was the equivalent of one thymus gland or liver/ml. of suspending medium.

Isolation of histones. Nuclei from a suspension in 10–12ml. were washed twice with 20ml. of 0·01M-tris–HCl–5mM-$MgCl_2$, pH 7·1 (Frenster, Allfrey & Mirsky, 1960), to remove nuclear ribosomes. The sediment was then washed two or three times with 20ml. of 1mM-HCl to remove low-molecular-weight nucleotides and was extracted with 5ml. of 50mM-HCl (Marsh, Ord & Stocken, 1964). A second extract with 20mM-HCl was rejected and the residue re-extracted with 5ml. of 250mM-HCl. The extracts in 50mM- or 250mM-HCl were clarified by centrifuging for 20–30 min. at 100000*g* (35000 rev./min. in a Spinco model L centrifuge, rotor SW 39).

Preparation of diffuse and dense chromatin. These were obtained from rat-thymus nuclei by the procedure of Frenster, Allfrey & Mirsky (1963). After preparing the nuclei in the usual way they were washed in tris–$MgCl_2$ and allowed to stand for 10min. at 0° with 0·25M-sucrose. The preparation was then treated ultrasonically for 1min. at 1·5A and 20000 cyc./sec. in an MSE Sonicator. The material was centrifuged for 10min. in a Servall centrifuge at 1000*g* to bring down the dense chromatin. The material that sedimented after a further 30min. at 3000*g* was discarded and diffuse chromatin was sedimented after 60min. at 80000*g* (35000 rev./min. in a Spinco model L centrifuge, rotor 40). After two or three washes with 1mM-HCl the histones were extracted from dense and diffuse chromatin in the normal way.

Separation of the histones. (*a*) Chemical methods. (1) In some experiments nuclei that had been washed with tris–HCl–$MgCl_2$ and with 1mM-HCl were extracted with 5% (v/v) $HClO_4$ (method 1 of Johns, 1964). The histone f1 was usually precipitated from such an extract by the addition of 100% (w/v) trichloroacetic acid to give a final concentration of 20%. The nuclear residue was further extracted with 50mM- and 250mM-HCl as described above, the extracts were made 1·25N with respect to HCl, 4 vol. of ethanol was added and the solutions were dialysed overnight against ethanol at 0°. The precipitates that formed in the dialysis sacs were separated by centrifugation and redissolved in 50mM- or 250mM-HCl. Then 3–5 vol. of acetone was added to the ethanolic supernatants and the resulting precipitates were also dissolved in 50mM- or 250mM-HCl.

(2) In other experiments the nuclei were extracted with 50mM- and 250mM-HCl in the usual way and these extracts made to 1·25N with respect to HCl. After the addition of 4 vol. of ethanol the solutions were dialysed against ethanol

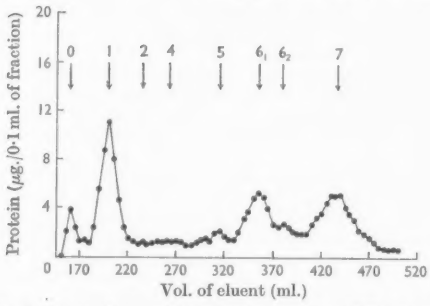

Fig. 1. Separation of histones from a 50 mM-HCl extract from rat thymus nuclei on Sephadex G-75. A 16–20 mg. sample of protein in 4–5 ml. was applied to a column (130 cm. × 2·5 cm. diam.) of Sephadex G-75. Fractions (5 ml.) were collected at a flow rate of 15 ml./hr. with 20 mM-HCl as eluent. The protein content was measured on 0·1 ml. from each tube by the method of Lowry *et al.* (1951). The peak numbers marked on the Figure correspond to those in Table 1.

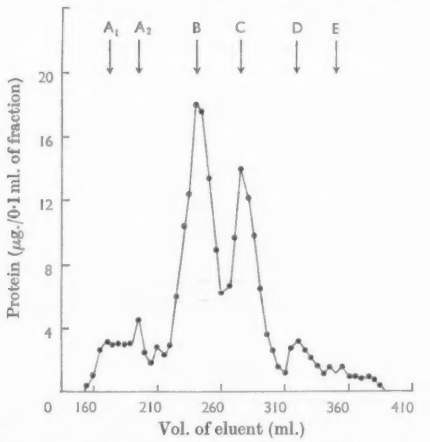

Fig. 2. Separation of histones from 250 mM-HCl extracts of liver or thymus nuclei on Sephadex G-75. A 16–20 mg. sample of protein in 4–5 ml. was applied to a column (130 cm. × 2·5 cm. diam.) of Sephadex G-75. Fractions (5 ml.) were collected at a flow rate of 15 ml./hr. with 20 mM-HCl as eluent. The protein content was measured on 0·1 ml. from each tube by the method of Lowry *et al.* (1951). (No differences were observed between the elution patterns of the extracts from liver and thymus.) The peak numbers marked on the Figure correspond to those in Table 1.

as in (1), and the ethanol- and the acetone-insoluble materials separated.

(3) Histones f3, f2a and f2b were obtained from thymus nuclei by method 2 of Johns (1964).

Table 1. *Histones detected by analytical electrophoresis in the peaks eluted from Sephadex G-75*

The main peaks were numbered as they were eluted from the Sephadex. Peak 1 was defined as the first big peak from the 50 mM-HCl extract which contained component 9; peak 7 was the peak from the 50 mM-HCl extract which contained only components 1–3. All the peaks were not found in every run and sometimes clearly separated minor peaks were present. Components in parentheses were present in trace amounts only. The components were identified by the position to which they moved after analytical electrophoresis under standard conditions. As far as could be ascertained the same component might be present in a number of different peaks and many were found in both the 50 mM- and 250 mM-HCl extracts.

50 mM-HCl extract		250 mM-HCl extract	
Peak	Components	Peak	Components
0	4, 5, 6, 7, 8	A_1	4, 5, 6, 7, 8, (10), (12)
1	4, 5, (6), 7, 8, 9	A_2	(6), 7, 8, (10), 12
2	4, 5, (6), 7, 8, 9	B	1–3, 4, 5, 7, 8, 10, 11, 12
3	(1–3), (4, 5), 7, 8, (9)	C	4, 5, 7, 8, 10, 12
(–)	(1–3), 4, 5, (6), (7, 8, 9)	D	4, 5, 7, 8, 10, (12)
4	1–3, 4, 5, (7, 8, 9)	E	4, 5, 7, 8, 10
5	1–3, 4, 5		
6_1	1–3, 4, 5, 7, 8		
(–)	1–3, 4, 5		
6_2	1–3, (4, 5)		
7	1–3		

(*b*) Exclusion chromatography. Some separation of the components in the 50 mM- and 250 mM-HCl extracts from rat-liver and -thymus nuclei was obtained by chromatography on Sephadex G-75 (Cruft, 1961; Johnson, Driedger & Marko, 1964). A column 130 cm. × 2·5 cm. diam. was used. The proteins were eluted with 20 mM-HCl; 5 ml. fractions were collected at a flow rate of 15 ml./hr. Figs. 1 and 2 show the peaks obtained from a 50 mM-HCl extract from thymus nuclei and from the 250 mM-HCl extract of both liver and thymus. The components detected in the different peaks after analytical electrophoresis are shown in Table 1.

Analytical electrophoresis on polyacrylamide gel. The histones present in the different fractions obtained by the chemical procedures or by chromatography were analysed by electrophoresis on polyacrylamide gel (McAllister, Wan & Irwin, 1963). Fig. 3 shows the components that have so far been distinguished, and their probable identification with the histones from calf thymus described by Johns (1964). The numbering of the bands agrees with that of the components detected by preparative electrophoresis (Ord *et al.* 1965). Where components 1–3 could not be distinguished they are referred to as fast-moving components, f_{mc}. About 75–80 μg. of proteins was applied to the gels and the electrophoresis was run for 2·5 hr. at 300 v and 3–4 mA/tube at pH 4·1 (37 mM-glycine–acetic acid buffer) at 20°.

Uptake of labelled precursors. A nuclear suspension (10–12 ml.) was incubated for 45 min. at 37° with 100 μc of ^{14}C-labelled protein hydrolysate (The Radiochemical

Centre, Amersham, Bucks.) (specific activity 600 μc/mg.) and 0·8 ml. of 0·9% NaCl containing 100 mg. of glucose/100 ml. At the end of the incubation the nuclei were washed five times with medium containing casein hydrolysate (1%) before extraction of the histones in the usual way.

In experiments on incorporation *in vivo* the ^{14}C-labelled amino acids, [8-^{14}C]adenine sulphate (28·9 mc/m-mole; The Radiochemical Centre) or inorganic [^{32}P]phosphate were injected intramuscularly (15 μc/100 g. body wt., 5 μc/100 g. and 50 μc/100 g. respectively) and the rats killed after 30–120 min.

Protein. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). The procedure was standardized by using dialysed HCl extracts from nuclei that were then dried *in vacuo* over P_2O_5.

Thiol and disulphide groups. These were measured by the method of Ellman (1959) as described by Marsh *et al.* (1964). 1-^{14}C-labelled *N*-ethylmaleimide (8 μc/mg.; Schwartz Bio-Research Inc., Mount Vernon, N.Y., U.S.A.) was used to label the thiol groups present in a 50 mM-HCl extract from thymus nuclei. The extract was brought to pH 4·5 and a 50% excess of *N*-ethylmaleimide added. After standing at 20° for 6 hr. the solution was dialysed overnight at 0° against 20 mM-HCl.

Phosphate determinations. Inorganic phosphate was measured by the method of Berenblum & Chain (1938). A final volume of 2 ml. of 2-methylpropan-1-ol–ethanol was used. The methods for acid-labile phosphates, total phosphate and for nucleic acid phosphorus were as described by Ord & Stocken (1961).

Serine phosphate. An 8 mg. sample of histone f1 was hydrolysed for 20 min. in 6 N-HCl at 100° as described by Schwartz & Lipmann (1961). After removal of the HCl *in vacuo* over KOH the hydrolysed material was dissolved in water and applied to a column (20 cm. × 0·4 cm. diam.) of Bio-Rad AG 50W (X8; 200–400 mesh) (Bio-Rad Laboratories, Richmond, Calif., U.S.A.). The column was eluted with water and 1 ml. fractions were collected. The fractions containing phosphate were freeze-dried and examined by ascending paper chromatography with propan-2-ol–10 N-HCl–water (65:16·6:18·4, by vol.) (Fink, Cline & Fink, 1963). Serine phosphate and threonine phosphate hardly separate in this solvent (R_F 0·53 and 0·55 respectively). The area containing the phosphate was eluted with water, and the eluate freeze-dried and hydrolysed in 6 N-HCl at 110° for 17 hr. (Crestfield, Moore & Stein, 1963). The HCl was removed under vacuum at 40° and the hydrolysed material from histone f1 and from the serine phosphate marker was examined by ascending paper chromatography in 2-methylpropan-2-ol–butan-2-one–water–aq. NH_3. (sp.gr. 0·88) (4:3:2:1, by vol.) (Fink *et al.* 1963), with serine and threonine as markers (R_F 0·38 and 0·54 respectively).

Ribose. Ribose was determined by the method of Dische & Schwartz (1937).

Deoxyribose. This was measured as described by Burton (1956).

Radioactivity measurements. The ^{14}C radioactivity was determined by gas-flow or scintillation counting (Nuclear-Chicago Corp. Tritomat; Isotope Developments Ltd., Beenham, Berks.). (The efficiency of the scintillation counter was 34%; that of the gas-flow counter was about 2%. Sufficient counts were recorded to give an error of less than 2%.) The ^{32}P radioactivity was measured in a liquid M6 Geiger–Müller tube or 2 ml. of the sample was placed within a glass tube surrounded by 5 ml. of scintillator and the activity measured in the Tritomat. The ^{32}P content of the bands separated by electrophoresis on polyacrylamide gel was determined by gas-flow counting as decribed by Marsh *et al.* (1964).

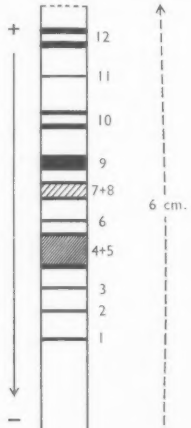

Fig. 3. Bands obtained after electrophoresis of 50 mM- and 250 mM-HCl extracts of rat-liver and -thymus nuclei on polyacrylamide gel. A 70–80 μg. sample of protein was applied to the gel (6 cm. × 0·4 cm. diam.). Electrophoresis was performed for 2½ hr. at 3 mA/tube and 300 v in glycine–acetic acid buffer, pH 4·1. The gels were stained with Naphthalene Black and destained electrophoretically. Bands 1–9 were commonly found in the 50 mM-HCl extracts and bands 4–8 and 10–12 in the 250 mM-HCl extracts. Band 10 probably corresponds to histone f3 of Johns (1964), band 9 to histone f1, and bands 4, 5, 7 and 8 to the components of histone f2.

RESULTS

Chemical separation of histones. In confirmation of previous findings (Ord *et al.* 1965) our results (Table 2) show that after incubation with ^{14}C-labelled amino acids *in vitro* the histones extracted from thymus nuclei with 50 mM-hydrochloric acid were of higher specific activity than those removed by 250 mM-hydrochloric acid. The 50 mM-hydrochloric acid extract was characterized by the presence of component 9 (histone f1 of Johns, 1964) and the relative absence of components 10 + 12 (histone f3); the 250 mM-hydrochloric acid extract normally contained no histone f1 but fairly large amounts of histones f3, f2a (components 7 + 8) and f2b (components 4 + 5) were present in both extracts. These four types of histone made up the greater part of the proteins extracted but components 1–3, which moved ahead of histone f2 on electrophoresis at pH 4·1, were also present and were relatively

Table 2. *Specific activity of different histones from rat-thymus nuclei obtained after chemical separation of the proteins*

Nuclei were isolated from rat thymus gland and incubated with ^{14}C-labelled protein hydrolysate for 45 min. at 37°. After washing the nuclei with medium containing casein hydrolysate (1%) the histones were extracted and precipitated as described in the text. Specific activity is expressed as counts/min./μg. of protein. Components in parentheses were present in trace amounts only.

	Method 1		Method 2	
	Probable components	Specific activity	Probable components	Specific activity
50 mM-HCl extract	—	—	1–9	66·9
250 mM-HCl extract	—	—	4, 5, 7, 8, 10, 12	19·5
5% $HClO_4$ extract	9	6·8	—	—
	1–3, 7, 8, 9	15·5	—	—
50 mM-HCl extract, ethanol ppt.	4, 5, (9), 10, 12	17·6	1–3, 4, 5, 6, 9, (10, 12)	81·6
		25·2*		48·4*
50 mM-HCl extract, acetone ppt.	7, 8, 10, 12	5·4	1–3, 7, 8, (10, 12)	35·7
				19·8*
250 mM-HCl extract, ethanol ppt.	4, 5, 10, 12	15·7	4, 5, 7, 8, 10, 12	41·5
		29·0*		25·9*
250 mM-HCl extract, acetone ppt.	7, 8, 10, 12	7·5	7, 8, 10, 12	12·8
		16·5*		11·3*

* These fractions were prepared from a batch of nuclei of which half was treated by method 1 and half by method 2.

Table 3. *Thiol contents of different histones from rat-thymus nuclei*

The nuclei were isolated and the histones extracted and precipitated as described in the text. The total thiol content was measured by the method of Ellman (1959) after reduction of any disulphide to thiol by borohydride. The total thiol content is given as μmoles of thiol plus disulphide/mg. of protein. Components in parentheses were present in trace amounts only.

Fractionation method	Components present	Total thiol content
Method 2		
50 mM-HCl extract, ethanol ppt.	1–3, 4, 5, 6, (7, 8), 9	37·0, 27·2
50 mM-HCl extract, acetone ppt.	1–3, (4, 5), 7, 8	3·3, 8·6
250 mM-HCl extract, ethanol ppt.	4, 5, (7, 8), 10, 12	36·0, 30·9
250 mM-HCl extract, acetone ppt.	(4, 5), 7, 8, (10, 12)	7·2, 8·8
Original 50 mM-HCl extract	1–9	37·0, 23·8
Original 250 mM-HCl extract	1–5, 7, 8, 10, 12	33·0, 10·3
Method 3: f3	10, 12	30·0
f1	9	0
f2b	4, 5	8·0

concentrated in the mixture of histones extracted with 50 mM-hydrochloric acid. The histone patterns obtained after electrophoresis, from nuclei before and after incubation at 37°, did not show any significant differences, suggesting that proteolysis during incubation did not account for the presence of relatively large amounts of components 1–3.

Precipitation with ethanol or acetone of the mixtures of histones already extracted with hydrochloric acid did not give clear-cut separation of the different components, although extraction of the nuclei with 5% perchloric acid usually gave fairly clean histone f1 (Johns, 1964). The specific activities of the different mixtures (Table 2), however, show that, in agreement with Laurence & Butler (1965), incorporation of amino acids into component 9 (histone f1) was relatively slow, as was that into components 10 + 12 (histone f3) and 7 + 8. Incorporation into components 4 + 5 was more rapid. When nuclei were extracted with 5% perchloric acid before further extraction with 50 mM- and 250 mM-hydrochloric acid much less clear separation between histones f1 and f3 was obtained and the amounts of components 1–3 appeared to be decreased, although this may have resulted from the presence of relatively large amounts of histones f1 and f3 in the extracts that were analysed by electrophoresis. It was also found that the material of very high specific activity was not extracted from nuclei if they were first treated with 5% perchloric acid (Table 2). This material, with high specific activity, was precipitated from

a normal 50mM-hydrochloric acid extract by method 2, and may correspond to band 6 on electrophoresis.

As previous results (Ord *et al.* 1965) had suggested that the fast-moving components on electrophoresis might be the thiol-containing histones (Marsh *et al.* 1964), the thiol plus disulphide contents of the different components obtained by precipitation were determined (Table 3). In agreement with the findings of Butler and his colleagues (Laurence & Butler, 1965; Phillips, 1965), histone f1 had no detectable thiol or disulphide groups, histones f2a and f2b contained some thiol plus disulphide groups and histone f3 was relatively sulphur-rich. The fairly large thiol content associated with the 50mM-hydrochloric acid extract appeared to be due to the presence of either component 6 or one or more of the fast-moving components.

Separation of the histones on Sephadex G-75. As the precipitation methods did not allow unequivocal separation of the different histones, an attempt was made to separate them by chromatography on Sephadex G-75 (Cruft, 1961; Johnson *et al.* 1964). Usually all the protein applied to the columns could be recovered and fairly good reproducibility was obtained between the different runs except that band 6, which in some experiments (Fig. 1) was in the first peak (0) off the column, was also obtained in a shoulder (2) immediately after the peak (1) containing a mixture of histones in which f1 predominated. As reported by Johnson *et al.* (1964) separation on Sephadex did not yield peaks containing single histones, although it was more satisfactory for the low-molecular-weight fast-moving components.

The pattern of incorporation of amino acids into histones extracted with 50mM-hydrochloric acid (Table 4) confirmed that obtained after differential precipitation. The presence of component 6 was again associated with material of high specific activity and uptake into histone f1 was low. When sufficient protein was available histone f1 could be separated from contaminants also present in the first large peak off the column by its solubility in 5% perchloric acid (Johns, 1964). Such treatment confirmed the low specific activity of this protein.

The specific activities of the histones after uptake of amino acids *in vivo* followed a very similar pattern to that found after uptake *in vitro*, and the distribution of specific activities between the histones of liver nuclei *in vivo* was very similar to that found in the histones from thymus (Table 4). The similarity of our results *in vivo* and *in vitro* suggest that the data are not invalidated by the observations of Sangster & Poort (1965) on the unsuitability of algal-protein hydrolysate as a source of ^{14}C-labelled amino acids for studies on incorporation *in vitro*. Qualitatively similar histones were extracted from the liver nuclei with 50mM- and 250mM-hydrochloric acid to those found from thymus but liver nuclei appeared to contain relatively more of components 7+8. This may have been due to the different method of preparation of the nuclei, but the electrophoretic behaviour of the histones extracted from thymus nuclei that had been prepared in 2·2M-sucrose medium was not significantly different from those obtained from nuclei made in 0·25M-sucrose medium.

When the histones from a 50mM-hydrochloric acid extract of thymus nuclei were labelled *in vitro* with ^{14}C-labelled *N*-ethylmaleimide the insignificant thiol content of histone f1 was substantiated (Table 4) and a fast-moving component and band 6 were found to be the thiol-rich components.

Incorporation of amino acids into the histones of the 250mM-hydrochloric acid extracts was also similar for liver and thymus *in vivo* (Table 5). The two small peaks of high-molecular-weight material eluted first from the columns were much more radioactive than the major peaks B and C. Electro-

Table 4. *Specific activities of different histones from rat-thymus and -liver nuclei obtained after separation of the histones from 50mM-hydrochloric acid extracts on Sephadex G-75*

The nuclei were isolated and the histones extracted and separated on Sephadex columns as described in the text. Expt. 1: specific activities of different peaks after thymus nuclei had been incubated with ^{14}C-labelled amino acids. Expt. 2: peaks from thymus nuclei after labelled amino acids had been injected into rats 1 hr. previously. Expt. 3: as Expt. 2, but with liver nuclei. Expts. 4 and 5: peaks from 50mM-HCl extracts from thymus nuclei, labelled *in vitro* with 1-^{14}C-labelled *N*-ethylmaleimide. The numbering of the peaks corresponds to that in Fig. 1, and the components present are shown in Table 1. Specific activities are expressed as counts/100sec./μg. of protein.

Expt. no.	Specific activity										
Peak...	0	1	2	3	–	4	5	6_1	–	6_2	7
1	—	27·5	21·5	8·7	20·9	—	1·25	—	1·03	—	—
2	—	0·81	5·65	2·38	—	—	0·86	—	0·81	—	0·96
3	—	0·35	1·18	0·46	—	0·69	—	—	—	—	—
4	—	4·5	19·4	—	—	—	—	7·5	—	7·4	—
5	50	4·2	—	23·4	—	34·8	—	16·9	—	38·4	20·5

Table 5. *Specific activities of different histones from rat-thymus and -liver nuclei obtained after separation of the histones from 250mM-hydrochloric acid extracts on Sephadex G-75*

The nuclei were isolated and the histones extracted and separated on Sephadex columns as described in the text. Expt. 1: specific activity after thymus nuclei had been incubated *in vitro* with ^{14}C-labelled amino acids. Expt. 2: peaks from thymus nuclei after labelled amino acids had been injected into rats 1hr. earlier. Expt. 3: as Expt. 2, but with liver nuclei. The numbering of the peaks corresponds to that in Fig. 2, and the components present are shown in Table 1. Specific activities are expressed as counts/100sec./μg. of protein.

Expt. no.	Specific activity					
Peak ...	A_1	A_2	B	C	D	E
1	—	—	24·1	27·3	—	—
2	1·71	1·47	0·69	0·69	0·99	0·86
3	1·71	3·12	0·33	0·27	—	—

Table 6. *Amino acid uptake and thiol plus disulphide contents of diffuse and dense chromatin obtained from rat-thymus nuclei*

Diffuse and dense chromatin were prepared as described by Frenster *et al.* (1963) and the histones extracted therefrom in the usual way. Conditions for amino acid uptake are described in the text. Thiol and total thiol plus disulphide contents are expressed as mμmoles/mg. of protein and ^{14}C uptake is expressed as $10^{-3}\times$ counts/min./mg. of protein.

	Dense chromatin			Diffuse chromatin		
	Histone f1	50mM-HCl extract	250mM-HCl extract	Histone f1	50mM-HCl extract	250mM-HCl extract
Amino acid uptake	12·0	20·2	9·4	37·0	38·7	26·0
SH	—	8·3*, 10·8†	11·5*, 28·0†	—	20·4*, 31·4†	15·4*, 29·0†
SH+S·S	—	15·6*, 21·2†	25·0*, 31·6†	—	26·9*, 31·4†	23·4*, 29·0†

* Expt. 1. † Expt. 2.

phoretic analysis of the protein of peaks A_1 and A_2 indicated the presence of traces of component 6.

Diffuse and dense chromatin. Frenster *et al.* (1963) described the greater rate of incorporation of a number of precursors into the RNA and protein of diffuse (active) chromatin as compared with dense (repressed) chromatin. Frenster (1965) has also established that the amount and type of histone obtained from the two chromatins are very similar. Our results on amino acid incorporation into the two types of chromatin (Table 6) and the pattern obtained after electrophoresis of the histones confirm Frenster's (1965) observations. When the thiol contents of the histones were examined (Table 6), however, the 50mM-hydrochloric acid extract from dense chromatin contained relatively more disulphide than the extract from diffuse chromatin. There may also have been rather less total sulphur. The total amounts of thiol in the 250mM-hydrochloric acid extracts did not differ. Dense chromatin is obtained much earlier in the preparation than diffuse chromatin, and histone extraction and thiol estimations were performed immediately so that preferential spontaneous oxidation of the thiol groups from dense chromatin seems unlikely. There was some indication that dense chromatin contained relatively more histone f1 than did active chromatin; this is substantiated by the observations of Littau, Burdick, Allfrey & Mirsky (1965) and explains the lower total sulphur content in the 50mM-hydrochloric acid extract from dense chromatin as histone f1 does not contain thiol or disulphide groups.

Incorporation of [^{32}P]phosphate into 50mM- and 250mM-hydrochloric acid extracts of nuclei. Examination of the spectrum of peak 0 (Table 1) showed that the protein(s) present absorbed in the ultraviolet with a broad shoulder at 255–280mμ (Fig. 4). As the extinction in the region/mg. of protein appeared to be considerably greater than for histone f1, which also does not show a prominent tyrosine peak, it seemed possible that nucleotide might be associated with component 6 either as a contaminant or bound to the protein.

To test this hypothesis [^{32}P]phosphate was injected intramuscularly 30–120min. before death and the nuclei from liver and thymus were washed an additional time with 1mM-hydrochloric acid to ensure removal of low-molecular-weight phosphate compounds. The radioactivity of all the washes was followed: ^{32}P was detected in both the 50mM- and 250mM-hydrochloric acid extracts from both sources of nuclei (Table 7). The radioactivity was not decreased by dialysis overnight against 50mM- or 250mM-hydrochloric acid, and when 230μg. of protein from a 50mM-hydrochloric acid extract

from thymus nuclei was analysed electrophoretically 13% of the counts remained at the origin, 27% was in the band associated with histone f1 and 53% was distributed over the region of histone f2 (components 4+5+7+8). The mean phosphorus contents of the 50mM- and 250mM-hydrochloric acid extracts were 26·0 (19·9–31·4) and 15·8 (8·4–22·6) mμg.atoms/mg. of protein respectively (five determinations). Histone f1 was also prepared (method 1 of Johns, 1964); on electrophoresis only very small traces of other proteins were detectable. The specific activity of ^{32}P-labelled histone f1 and its ^{31}P content were unchanged by dialysis. It contained 23·4 (20·4–27·8) mμg.atoms of P/mg. of protein (five determinations).

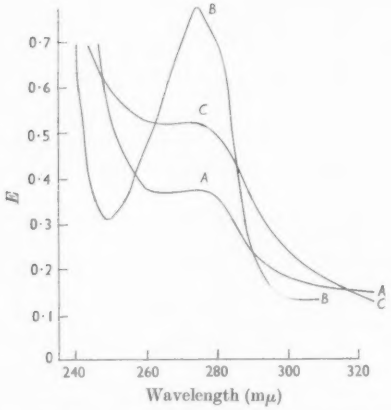

Fig. 4. Absorption spectrum of histones obtained from a 50mM-HCl extract of rat-thymus nuclei. Histone f1 (component 9) was obtained by method 1 of Johns (1964) and histone f2a by method 2. Peak 0 was obtained after separation of a 50mM-HCl extract of rat-thymus nuclei on Sephadex G-75. The spectra were examined in the range 320–240mμ in a Beckman DK–2A spectrophotometer. The protein concentrations were: histone f1 (*A*), 1·30mg./ml.; histone f2a (*B*), 1·94mg./ml.; peak 0 (*C*), 0·24mg./ml.

Because of the high radioactivity of many of the phosphorylated derivatives in nuclei, the extent of ^{32}P incorporation into a number of phosphates was followed over a 2hr. period. Previous experiments (Ord & Stocken, 1961) had shown the presence of inorganic phosphate which was bound to thymus nuclei, but which could be removed with cold dilute acid. N-Perchloric acid was used and it was noted that a small amount of phosphorus in this extract was associated with a soluble protein which was precipitated by phosphomolybdate in the procedure of Berenblum & Chain (1938) for determining inorganic phosphate. It is now believed that the protein precipitated under these conditions was histone f1.

The time-course of ^{32}P uptake into bound inorganic phosphate, acid-labile phosphate, RNA and DNA suggested (Table 7) that the radioactivities of histone f1 and the 50mM-hydrochloric acid extract were unlikely to be due to contamination from the types of nucleic acid phosphorus examined. The distribution of counts through the preliminary washes showed that ^{32}P radioactivity was removed with tris–magnesium chloride and

Table 7. *Specific activity of phosphorylated compounds from rat-thymus nuclei*

[^{32}P]Phosphate was injected intramuscularly (50μc/100g. body wt.) 30–120min. before death. Nuclei were isolated and extracted as described by Marsh *et al.* (1964). Histone f1 was prepared by the method of Johns (1964). The specific activities of bound inorganic phosphate and acid-labile phosphate were recalculated from the data of Ord & Stocken (1961). Ribosomal RNA was the RNA extracted from the nuclei with 0·01 M-tris–HCl–5mM-$MgCl_2$, pH 7·1 (Frenster *et al.* 1960), and residual RNA was RNA remaining in the nuclei when ribosomal RNA, acid-extractable proteins and lipids had been removed. Specific activities are expressed as counts/100sec./μg. of phosphorus.

Expt. no. (Ord & Stocken, 1961)	Time after injection (min.)	Specific activity 30	60	120
	Bound inorganic P	990	648	512
	Acid-labile P	610	521	501
1	Ribosomal RNA P	2	3	3
1	Residual RNA P	7	26	31
1	DNA P	2	9	15
1	Histone f1	46	56	73
2	Histone f1	—	223	195
3, 4, 5*	Histone f1	29	188	79
3, 4, 5*	50mM-HCl extract	6	65	64
3, 4, 5*	250mM-HCl extract	14	15	36

* In Expts. 3–5, each of the time-periods represents a different experiment, but histones f1 and the 50mM- and 250mM-HCl extracts were all prepared from the same batch of nuclei.

that the washes with 1mM-hydrochloric acid took out progressively less and less activity; 5% perchloric acid of 50mM-hydrochloric acid markedly increased the amount of ^{32}P extracted but the results after dialysis appeared to exclude contamination of the proteins by low-molecular-weight compounds.

At 60min. after injection the uptake of ^{32}P into the histones of the 50mM-hydrochloric acid extract considerably exceeded that into the histones of the 250mM-hydrochloric acid extract (Table 7), and this is supported by the radioactivities of the proteins after separation on Sephadex G-75 (Table 8). In the second experiment with thymus component 6 was found by electrophoresis to have separated in peak 0 just ahead of the first big peak, and had a lower specific activity/μg. of P than the other components of the 50mM-hydrochloric acid extract, although its activity/mg. of protein was higher. The behaviour of the 50mM-hydrochloric acid extract from liver nuclei was similar to that from thymus although the activity/μg. of P was higher throughout.

Nature of the phosphate groups. In examining the nature of the phosphate group in histone f1 and in the whole 50mM-hydrochloric acid extract the possibilities considered were that the phosphate, either as inorganic phosphate or as nucleotide phosphate, was covalently bound to the proteins, or that there was a particularly stable salt formed between the proteins and the ester phosphate. Because of the small amounts of material available [^{14}C]adenine uptake *in vivo* was investigated to see whether adenine was present in any of the fractions. Histone f1 and the 50mM- and 250mM-hydrochloric acid extracts were prepared in the usual way from liver and thymus nuclei 2hr. after injection. Uptake into extracts from liver nuclei was much higher than into those from thymus (thymus: 50mM-hydrochloric acid extract, 2·5counts/min./mg. of protein; 250mM-hydrochloric acid extract, $\pm$; liver: 50mM-hydrochloric acid extract 15·5; 250mM-hydrochloric acid, 4·7). No counts were detected in histone f1 from either source.

The absence of nucleotide from histone f1 allowed the possibility that the phosphate might be present as serine phosphate or threonine phosphate. ^{32}P-labelled histone f1 was prepared and hydrolysed for 20min. in 6N-hydrochloric acid at 100° (Schwartz & Lipmann, 1961). The phosphate was not liberated as inorganic phosphate but remained as organophosphate. After chromatography on Bio-Rad AG50W (see the Methods section), ninhydrin-positive material was not detected in the first two fractions, which contained the ^{32}P. Ascending paper chromatography of the phosphorus-containing material showed that the ^{32}P migrated in the same position as an authentic sample of serine phosphate. The ^{32}P-containing area and that of the authentic sample of serine phosphate were eluted, hydrolysed overnight in 6N-hydrochloric acid at 110° and rechromatographed. The material obtained from histone f1 behaved identically with that from authentic serine phosphate. No sign of threonine was detected.

The [^{14}C]adenine uptake into the 50mM- and 250mM-hydrochloric acid extracts suggested that their phosphorus contents might be due to contamination from nucleotide material other than that examined above. Treatment of the 50mM-hydrochloric acid extract with ribonuclease or with phenol (Kirby, 1956) failed to decrease the phosphorus content nor was it removed by passing the proteins through a column of Dowex 1 (Cl^- form). When the phosphorus contents of histones prepared from thymus nuclei after extraction with 50mM-hydrochloric acid and dialysis against ethanol were compared with those obtained by method 2 of Johns (1964), ^{31}P and ^{32}P were detected mainly in the precipitate from the 50mM-hydrochloric acid extract dialysed against ethanol and in histones f1 and f3. Histones f2a and f2b did not contain

Table 8. [^{32}P]*Phosphate uptake by proteins extracted from liver and thymus nuclei with* 50mM- *and* 250mM-*hydrochloric acid*

[^{32}P]Phosphate was given to the rats 60min. before death. The nuclei were washed and the proteins extracted and separated on Sephadex columns as described in the text. The components in the peaks were identified by electrophoresis on polyacrylamide gel, and the peaks numbered as in Figs. 1 and 2. ^{32}P radioactivity is expressed as counts/10^3sec. per mg. of protein or per μg. of phosphorus.

		50mM-HCl extract						250mM-HCl extract		
	Peak.....	1	2	3	5	6	7	B	C	D
^{32}P uptake/μg. of P	Liver	2970	—	5290	2640	—	—	—	—	—
	Thymus: 1	1875	1350	1270	—	1370	—	—	—	—
	2	1882	447	—	1242	1140	2115	97	75	149
^{32}P uptake/mg. of protein	Liver	2860	—	927	—	—	—	—	—	—
	Thymus: 1	1376	1760	1226	—	240	—	—	—	—
	2	1350	2440	—	1115	584	1349	237	111	795

phosphorus. Since extraction of nuclei with 50mM-hydrochloric acid or higher acid strength appeared to remove a phosphorus compound in either method, it seemed that the phosphorus component(s) other than histone f1 might be extracted in either procedure and become associated with the other proteins present in the extracts.

A strong similarity was observed between the behaviour of this phosphorus compound, the component into which rapid uptake of amino acids occurred and the thiol-rich material examined above. This was confirmed in an experiment in which the histones from thymus nuclei were labelled with ^{14}C-labelled amino acids and [^{32}P]-phosphate *in vivo* and the phosphorus and thiol contents of the 50mM-hydrochloric acid extract were followed (Table 9). Both the thiol groups and the phosphorus appeared to be associated with material extracted in 50mM-hydrochloric acid that was precipitated by dialysis against ethanol, was not removed by dialysis against hydrochloric acid and was not histone f1. The amounts of phosphorus and of thiol plus disulphide were equimolar and were also associated with protein having an increased uptake of amino acids. It was not possible from the electrophoretic analyses to define precisely the component with the appropriate properties. Component 6 did not separate in any of these analyses.

The separation of the [^{14}C]adenine-labelled extract from thymus nuclei on Sephadex G-75 referred to above gave a small peak 0 containing [^{14}C]adenine radioactivity and ^{31}P, which was eluted before the first big peak. When this peak 0 was analysed for ribose by the orcinol method (Dische & Schwartz, 1937) a brown colour was produced, masking any possible ribose content, and probably being attributable to glucose derived from oligosaccharide. Oligosaccharide was also detected in peak 7, the only other material to give low, but unequivocal, [^{14}C]adenine radioactivity. Thymus nuclei are known to contain glycogen (Ord & Stocken, 1962). It seems possible therefore that the thiol- and phosphorus-containing compound extracted from nuclei with 50mM-hydrochloric acid becomes associated with oligosaccharide or low-molecular-weight basic protein or both and distributes itself on electrophoresis or on Sephadex according to the properties of its associated molecule.

Hydrolysis of peak 0 in 6N-hydrochloric acid overnight at 110° and chromatography of the hydrolysed material in the butan-2-one solvent showed the presence of some material absorbing in the ultraviolet and five spots reacting with ninhydrin. Three of these have been tentatively identified as threonine, serine and glutamic acid or aspartic acid.

DISCUSSION

The rates of uptake of amino acids into the main histones extracted from liver and thymus nuclei confirm observations of Laurence & Butler (1965) that there is little difference in the rapidity of synthesis of these proteins, which are renewed at a detectable rate. The greater incorporation of amino acids into the histones extractable from diffuse chromatin compared with those from dense chromatin also confirms observations by Frenster *et al.* (1963). The same amount of histone is found

Table 9. *Phosphorus and thiol contents of proteins from a 50mM-hydrochloric acid extract from thymus nuclei*

Rats were given 25μC of [^{32}P]phosphate and 17·7μC of ^{14}C-labelled amino acids/120g. body wt. 60min. before death. The 50mM-HCl extract was obtained in the usual way. It was then dialysed against 100 vol. of ethanol and the precipitate redissolved in 50mM-HCl and made 0·74N (5%) with respect to $HClO_4$. The resulting precipitate was dialysed against two changes of 10 vol. of 50mM-HCl. ^{31}P is expressed as mμg.atoms/mg. of protein; SH (or SH+S·S) is expressed as mμmoles/mg. of protein. ^{32}P radioactivity is expressed as counts/10^3sec./mg. of protein, determined by liquid counting; ^{14}C radioactivity is expressed as counts/10^3sec./mg. of protein, determined by scintillation counting.

	^{32}P	^{14}C	SH	^{31}P	Components present
Original 50mM-HCl extract	215	9590	25·9	—	1–9
Ppt. after dialysis against ethanol	371	11340	30·7	—	—
Material not pptd. after dialysis against ethanol	84	8320	—	—	4, 5, 7, 8, (9)
Ethanol-insoluble material pptd. in 0·74N-$HClO_4$	329	12440	27·0 37·6*	36·8	2, 3, 4, 5, 9
Ethanol-insoluble material not pptd. by 0·74N-$HClO_4$	246	8700	0	23·4†	2, (3), (7, 8), 9

* SH+S·S.

† From previous determinations in this paper.

in these two types of chromatin (Frenster, 1965) and our results support the view that the histones present in these two kinds of chromatin are qualitatively identical, although relatively more histone f1 may be located in dense chromatin. The greater proportion of disulphide in dense chromatin could contribute to the cross-linking of lysine-rich histones between double helices of DNA suggested by Littau *et al.* (1965) and indicates that alterations in thiol/disulphide balance may be linked with alterations in the rate of synthesis of associated components.

Unfortunately, we are still unable to define precisely the nature of the thiol compound. The present results suggest that, although the material is extracted together with basic protein, of the histones obtained by the second procedure of Johns (1964) only histone f3 contains thiol groups, and it may be that this sulphur is only present because it is extractable from nuclei under the same conditions as histone f3 and becomes linked with it. If nuclei are extracted with 50mM-hydrochloric acid histone f3 is not removed but a considerable proportion of the thiol is, and now becomes associated with the basic proteins of this extract.

The presence of small amounts of phosphorus in the acid extracts was unexpected and it is difficult to eliminate entirely the possibility that it may be due to fragments of messenger RNA which either are, or become, attached to histone. The linkage of the phosphorus with the protein is not random and phosphorus is not present in histone f2a or f2b. The parallel properties of the phosphorus and thiol components and of the material with higher uptake of amino acids are striking and lend support to the view that nuclei possess a fairly rapidly synthesized thiol- and nucleotide-containing peptide. Such nucleotide peptides containing cysteine have been described in liver (Steinberg, Vaughan, Sherman & O'Dell, 1960; Chang & Wilken, 1965). Separation of histones on Sephadex is not clear-cut, but the data on adenine and amino acid uptake and thiol and phosphorus contents of the different peaks are consistent with the presence of a thiol- and phosphate-containing compound in association with oligosaccharide of mol.wt. at least 40000 and with proteins 1–3, which move ahead of histone f2 on electrophoresis. Until its properties are substantiated it is only possible to speculate on the significance of such a compound in nuclear metabolism.

The presence of phosphate on one of the serine residues of histone f1 is more clearly established. Frenster (1965) has discussed the possible functions of nuclear phosphoproteins in binding histones and so preventing repression of RNA synthesis. It would be surprising if one phosphate group in addition to the other carboxylic acid residues in histone f1 affected its capacity to bind nucleic acids but the presence of phosphorylated protein in acid extracts from nuclei may be a complication for other work on nuclear metabolism.

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The Tricarboxylic Acid Cycle and Glycolysis in Relation to Ion Transport by the Ciliary Body

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1. The respiration and aerobic glycolysis of pig ciliary processes in oxygenated phosphate and bicarbonate buffers have been investigated. 2. Significant amounts of lactic acid are produced only in the presence of added glucose, but this does not change the endogenous respiration rate. 3. Succinate and citrate increase the oxygen uptake considerably, but pyruvate has almost no effect; oxaloacetate and fumarate stimulate slightly in the presence of glucose. Aspartate and fumarate together stimulate pyruvate utilization and are oxidized as fast as citrate. 4. Ouabain inhibits the oxidation of glucose and other substrates by limiting the ADP supply from the sodium transport system. Cyanide and azide inhibit respiration and stimulate glycolysis. 5. The transport mechanism depends largely on ATP from oxidative phosphorylation and regulates the rate of respiration and glycolysis by controlling ADP production from the Na^+–K^+-activated adenosine triphosphatase.

The formation of aqueous humour depends on the movement of Na^+ ions from the blood capillaries, against an electrochemical gradient across the ciliary epithelium, into the posterior chamber of the eye (Cole, 1961*a*). It has been shown by de Roetth (1953) that the ciliary body has a high rate of aerobic glycolysis, a characteristic apparent elsewhere among normal adult animal tissues only in the retina. It seemed therefore that ion transport in this tissue might be geared to ATP generated by glycolysis, or by oxidative phosphorylation, or both. However, experiments *in vitro* with cyanide, 2,4-dinitrophenol and under anaerobic conditions suggested that sodium transport across the epithelium depended very largely on oxidative metabolism (Cole, 1961*b*), and that energy derived solely from glycolysis was unable to support a high rate of sodium movement.

In erythrocytes, where sodium transport depends entirely on glycolysis, it has been shown that inhibition of transport by ouabain causes a marked decrease in the rate of glycolysis (Whittam, Ager & Wiley, 1964). A similar and even more marked inhibition can be demonstrated in the ciliary body, indicating a definite relation between the glycolytic and transport processes (Riley, 1965). It is therefore of interest to determine whether the unusual metabolism of the ciliary body is directly related to its function of aqueous humour formation. Preliminary accounts have already been published (Riley & Baroncelli, 1964; Riley, 1964).

MATERIALS AND METHODS

Pig eyes were obtained from freshly killed animals; the eyes were transported in crushed ice and used within 4 hr. of death. The ciliary body and iris were removed together from the anterior segment and then the iris and most of the posterior part of the ciliary body were cut away, leaving a thin circle of tissue about 2 mm. thick consisting largely of ciliary processes, of about 70 mg. wet wt. These preparations were immersed in ice-cold 0·3 M-mannitol until required.

The incubating medium was of pH 7·4 and contained: PO_4^{3-}, 21 mM; Cl^-, 117 mM; Na^+, 125 mM; K^+, 30 mM; Mg^{2+}, 1·2 mM. Glucose was added to give 3·3 mM final concentration and other substrates were 10 mM. In some experiments Krebs–Ringer bicarbonate medium (Umbreit, Burris & Stauffer, 1957) was used, uptake of O_2 being measured by the indirect method of Warburg. Ouabain, used at 0·1 mM, was obtained from L. Light and Co. Ltd., Colnbrook, Bucks. Sodium-free media were prepared by using tris–HCl in place of NaCl–KCl and K_2HPO_4 in place of Na_2HPO_4; the final composition was: PO_4^{3-}, 21 mM; Cl^-, 117 mM; tris, 117 mM; K^+, 32 mM; Mg^{2+}, 1·2 mM; pH 7·4. Tissues were incubated individually in 1·5 ml. of medium in 10 ml. Warburg flasks for a period of 40 min. at 37°, with a gas phase of 100% O_2, or $O_2 + CO_2$ (95:5) when bicarbonate buffer was used. Glucose was measured by the glucose oxidase method (Marks, 1959) and lactate by the method of Barker & Summerson (1941). The glucose and lactate content of aqueous extracts of the tissue were measured in some experiments, but there was no appreciable change in either quantity during incubation, and results presented refer only to quantities found in the medium.

Extracts of tissue incubated in the presence of [U-^{14}C]-

Table 1. *Oxygen uptake, glucose utilization and lactic acid production by pig ciliary processes in the presence of glucose and tricarboxylic acid-cycle intermediates*

Experimental details are given in the text. Results are presented as means ±S.E.M. with the numbers of experiments in parentheses.

Substrate added	Uptake of O_2 (μl./100 mg. wet wt./hr.)	Glucose utilized (μmoles/100 mg. wet wt.)	Lactate produced (μmoles/100 mg. wet wt.)	Ratio lactate produced: glucose utilized
None	93±2·5 (4)	<0·1	0·2±0·1 (4)	—
Glucose	91±3·3 (6)	2·8±0·2 (6)	4·9±0·3 (6)	1·7
Succinate	174±8·6 (6)	<0·1	0·3±0·1 (4)	—
Citrate	144±4·9 (6)	—	—	—
Glucose+succinate	177±3·9 (8)	2·3±0·1 (4)	3·5±0·5 (4)	1·5
Glucose+citrate	149±2·0 (6)	2·6±0·2 (4)	3·6±0·2 (4)	1·4
Glucose+pyruvate	103±4·9 (5)	1·2±0·2 (6)	3·9±0·2 (6)	3·3
Glucose+oxaloacetate	129±2·9 (6)	2·2±0·1 (4)	2·5±0·2 (4)	1·1
Glucose+pyruvate+oxaloacetate	130±3·1 (9)	2·0±0·1 (4)	2·7±0·4 (4)	1·4
Glucose+fumarate	118±3·4 (5)	1·8±0·2 (6)	3·2±0·3 (4)	1·8
Glucose+pyruvate+fumarate	114±3·1 (5)	1·2±0·2 (5)	3·7±0·3 (5)	3·1
Glucose+fumarate+aspartate	145±3·3 (6)	2·4±0·2 (4)	3·2±0·4 (4)	1·3
Glucose in Krebs–Ringer bicarbonate buffer	94±5·1 (3)	2·9±0·4 (3)	5·1±0·6 (3)	1·8

glucose (2 μc/flask) were made by homogenizing in 2 ml. of 30% (v/v) ethanol. These extracts were freeze-dried and resuspended in 0·1 ml. of water. Spots (50 μl.) were applied to Whatman no. 1 papers (40 cm.×40 cm.), which were then developed in butan-2-ol–formic acid–water (5:1:1, by vol.) for 14 hr., followed in the second dimension by phenol–water–aq. NH_3 soln. (80:20:1, by vol.) for 16 hr. After drying the papers for 48 hr. radioautographs were made by using Kodak Industrex D X-ray film, with an exposure of 10–12 days.

RESULTS

Oxidation of substrates in phosphate and bicarbonate buffers. The respiratory activity of ciliary body in the presence of glucose and several substrates of the tricarboxylic acid cycle is shown in Table 1. Although glucose did not increase the oxygen uptake above that found in the absence of added substrates, it greatly increased the amount of lactic acid produced. It can be seen that about 85% of the glucose utilized was converted into lactate, whereas the remaining 15%, which by the use of [^{14}C]glucose was shown to be metabolized beyond pyruvate to carbon dioxide, would account for the observed oxygen uptake. The endogenous substrates have not been identified but it appears that in the presence of other substrates they were not preferentially utilized.

Citrate and succinate produced a large stimulation of respiration whether glucose was present or not. Glucose utilization was depressed only slightly and 70–80% was still converted into lactate. Pyruvate stimulated oxygen uptake only slightly but depressed glucose utilization very strongly. The lactate:glucose ratio was high, as a result of conversion of exogenous pyruvate into lactate by NADPH formed in the hexose monophosphate pathway, which is stimulated under these conditions (Kinoshita, 1957; Riley & Baroncelli, 1964). Fumarate and oxaloacetate both stimulated oxygen uptake but not to the extent of citrate. The addition of pyruvate with either of these two substrates in the presence of glucose caused no appreciable change in the rate of oxygen uptake. It is apparent from the lactate:glucose ratios that fumarate did not cause the pyruvate resulting from glycolysis to be diverted into the cycle, whereas oxaloacetate, although depressing glucose utilization almost as much as fumarate, caused a much greater amount of pyruvate to be oxidized via the cycle. Therefore it seemed that although fumarate was oxidized, the product was not able to stimulate pyruvate utilization. Each of the substrates added to the medium does cause a large fall in the amount of $^{14}CO_2$ produced from [^{14}C]glucose (Riley, 1964), indicating that all substrates entered the cells in the present experiments.

On incubation of the ciliary body with [U-^{14}C]-glucose, a considerable quantity of the label was found in aspartate, only lactate and glutamate showing higher radioactivities on the chromatogram. Specific activities were not measured, but it appears that appreciable amounts of cycle intermediates may be converted into their corresponding amino acids. On addition of aspartate to the ciliary body oxidizing fumarate and glucose, it was found that the rate of oxygen uptake was equal to that of citrate, and that endogenous pyruvate utilization was increased in a manner similar to that when oxaloacetate was present (Table 1). Therefore there can be no limitation to the oxidation of pyruvate by pyruvate oxidase or the condensing

enzyme, the rate being determined by the amount of cycle intermediates being made available as oxaloacetate. Exogenous oxaloacetate was not as effective as aspartate in supporting a high rate of oxidation because at the concentration used, 10mM, it caused a small inhibition of citrate oxidation.

Incubation of ciliary body in Krebs–Ringer bicarbonate with a gas phase oxygen+carbon dioxide (95:5) did not increase the rate of oxygen uptake with glucose, or other substrates, above that observed in the phosphate buffer (Table 1). Thus it seems unlikely that ciliary body is capable of increasing the supply of cycle intermediates by means of the malate reaction, as has been postulated for the retina (Kornblueth, Yardeni-Yaron & Wertheimer, 1953).

Effects of cyanide and azide. The effects of cyanide and azide on the respiration and glucose utilization of the ciliary body are shown in Table 2. About 20% of the respiration was found to be cyanide-insensitive, in agreement with de Roetth (1953); azide inhibited up to 55% at a concentration of 10mM, and the amount of glucose converted into lactate was greatly increased by both inhibitors at 1mM. If the sum of ATP synthesized by glycolysis and oxidative phosphorylation is calculated by the method of Quastel & Bickis (1959), with 1mM-azide the value is the same as the controls, whereas with 1mM-cyanide the value is much lower than the controls (Table 2).

Omission of sodium from the medium. The replacement of Na^+ ions in the incubating medium by tris caused a marked inhibition of respiration and glycolysis of ciliary body utilizing glucose (Table 3). Similar results were obtained when ouabain was added to the medium instead of removing Na^+ ions, but no further inhibition of respiration or glycolysis could be obtained by adding ouabain to the medium lacking Na^+ ions. Under anaerobic

Table 2. *Effect of cyanide and azide on the respiration and aerobic glycolysis of pig ciliary processes incubated with glucose*

Experimental details are given in the text. Yield of ATP is calculated by the method of Quastel & Bickis (1959). Each value is the mean of at least three experiments.

Inhibitor	Uptake of O_2 (μmoles/100mg. wet wt./hr.) (a)	Lactate produced (μmoles/100mg. wet wt./hr.) (b)	Calc. yield of ATP ($6a+b$)
None	3·8	7·6	30·4
CN^- (1mM)	0·7	12·6	16·8
N_3^- (1mM)	2·8	12·5	29·3
N_3^- (2·5mM)	2·1	—	—
N_3^- (10mM)	1·7	—	—

Table 3. *Effect of ouabain or absence of Na^+ ions on respiration and glycolysis of pig ciliary processes under different conditions*

Experimental details are given in the text. Each value is the mean of at least four experiments except where indicated by numbers in parentheses. C, Control flasks; E, flasks with ouabain present or with sodium replaced by tris; I, percentage inhibition found in the experimental flasks.

Conditions		Uptake of O_2 (μl./100mg. wet wt./hr.) C	E	I (%)	Glucose utilized (μmoles/100mg. wet wt./hr.) C	E	I (%)	Lactate formed (μmoles/100mg. wet wt./hr.) C	E	I (%)
Aerobic	Ouabain	85	31	63	4·6	2·2	52	7·1	2·6	63
	Zero Na^+	85	33	61	4·6	2·4	48	7·1	3·0	58
	Zero Na^++ouabain (1)	88	35	60	—	—	—	7·4	2·9	61
Anaerobic	Ouabain	—	—	—	5·5	3·2	42	10·4	5·8	44
	Zero Na^+	—	—	—	5·5	3·5	36	10·4	6·3	39
With dinitrophenol (20μM)										
Aerobic	Ouabain	147	72	51	5·3	3·0	43	7·7	4·9	36
	Zero Na^+ (2)	139	67	52	5·0	3·0	40	7·0	4·7	33
Anaerobic	Ouabain	—	—	—	6·0	4·4	27	11·2	9·2	18

Table 4. *Effect of ouabain on respiration of pig ciliary processes oxidizing succinate or citrate in the presence or absence of glucose*

Experimental details are given in the text. Results are presented as means ±S.E.M. with the numbers of experiments in parentheses.

Substrate	Uptake of O_2 (μl./100mg. wet wt./hr.) Control	With 0·1 mM-ouabain	Inhibition due to ouabain (%)
Succinate	174±8·6 (6)	67±3·2 (6)	61
Succinate+glucose	175±4·5 (6)	77±2·7 (6)	56
Citrate	144±4·9 (6)	33±2·0 (6)	77
Citrate+glucose	149±2·0 (6)	38±1·6 (6)	74

conditions or in the presence of 2,4-dinitrophenol the inhibition of glycolysis is considerably less than under aerobic conditions. Table 4 shows that the oxidation of succinate and citrate is severely inhibited by ouabain. As with glucose oxidation, removal of Na^+ ions from the medium caused an effect comparable with that of the cardiac glycoside. The smaller inhibition of succinate oxidation as compared with citrate oxidation under these conditions reflects the lower degree of respiratory control that is always observed in succinate metabolism (Hatefi, Jurtshuk & Haavik, 1961). The results have been presented to show the small but significant difference ($P < 0.05$, Student's t test) between the ouabain-inhibited succinate oxidation rate in the presence and absence of glucose. The same effect, but smaller and not significant in the samples tested, can be seen with citrate oxidation.

DISCUSSION

The results presented in Table 1 show that the rate of pyruvate oxidation by the ciliary body is not limited by its rate of oxidation to acetyl-CoA, nor by the rate of reaction after condensation with oxaloacetate to form citrate. Pyruvate must be made available to the site of the tricarboxylic acid cycle in excess of its rate of removal by this path, the excess being converted into lactate. As each of the intermediate substrates between citrate and oxaloacetate has been found to be oxidized at a higher rate than pyruvate, the limiting factor in pyruvate oxidation must be the availability of oxaloacetate. From the experiments with [^{14}C]-glucose it appears that cycle intermediates can be transaminated to α-oxoglutarate and oxaloacetate. When aspartate is provided in the incubation medium to prevent this 'loss' of oxaloacetate, then pyruvate is metabolized as fast as citrate, and the turnover of the cycle must be determined by the availability of ADP, as dinitrophenol has been shown to almost double the rate of oxygen uptake by the ciliary body utilizing glucose (Table 3). The 'loss' of oxaloacetate to form aspartate is significant only when the rate of formation of oxaloacetate is low, as when ADP is limiting. Dinitrophenol stimulates turnover of the cycle to such an extent that additional oxaloacetate is not necessary to ensure maximum rate of condensation with acetyl-CoA.

de Roetth (1953) found a respiratory quotient 0·95 for ox ciliary body, indicating a predominantly carbohydrate source of metabolites and, as the ciliary body has an abundant blood supply extending right to the tips of the processes (Scullica, 1962), it is probable that glucose is the normal substrate of the epithelial cells. On the basis of the present experiments *in vitro* therefore it can be calculated that about 75% of the ATP produced in the ciliary epithelium arises from oxidative metabolism in the mitochondria (ratio of glucose oxidized to that converted into lactate, 1:6; ratio of aerobic to anaerobic ATP yield, 18:1; therefore the ratio of aerobic ATP to glycolytic ATP is 3:1). Does the remaining 25% of available energy play a specific role in sodium transport and, if not, is the high rate of aerobic glycolysis essential for the formation of aqueous humour?

Ouabain, which inhibits Na^+–K^+-activated adenosine triphosphatase (Skou, 1960), gives some measure of the fraction of metabolic activity which depends on a supply of ADP via this enzyme, and how much is provided by other reactions resulting in ADP formation; the stimulation of oxidation of succinate in the presence of ouabain by addition of glucose (Table 4), thereby causing utilization of ATP, indicates the importance of other sources of ADP when the Na^+–K^+-activated adenosine triphosphatase is inhibited. Thus, in the ciliary body, where sodium transport may be assumed to be a major energy-requiring role of the tissue, inhibition of oxygen uptake is about 60% as compared with 40% and 30% respectively in the liver and brain, in which both synthetic reactions and transport processes may be expected to have a need for ATP (Whittam & Willis, 1963; Elshove & van Rossum, 1963).

The inhibition of lactate formation by ouabain

in the ciliary body under aerobic conditions is much higher than that observed in erythrocytes. However, under anaerobic conditions the degree of inhibition is considerably decreased, from 63% to 44%, and in the presence of dinitrophenol the effect of ouabain is diminished even more under both aerobic and anaerobic conditions. These observations suggest that a competition exists between the mitochondria and soluble fraction of the epithelial cell for the available ADP, resulting, under aerobic conditions, in a more severe inhibition than is observed in erythrocytes which have no mitochondria to compete for ADP.

Glycolysis in the ciliary body appears to depend on a supply of ADP from the Na^+–K^+-activated adenosine triphosphatase. Similar observations have been made in erythrocytes (Whittam *et al.* 1964) and in freshwater-turtle bladder (Klahr & Bricker, 1965) and in each of these cases it has been shown that sodium transport can be maintained by glycolytic ATP. This is not true for the ciliary body, where oxidative metabolism is obligatory for a normal rate of transport (Cole, 1961*a*,*b*). However, the experiments of Cole (1961*b*) indicated that transport processes were depressed to a lesser extent by dinitrophenol, cyanide and anoxia than by ouabain, suggesting that a diminished rate of sodium transport can be supported by glycolytic ATP. That anoxia causes a greater fall in the epithelial potential than cyanide (Cole, 1961*b*) is in agreement with this concept, as the greater stimulation of glycolysis by cyanide provides more ATP to support transport than is available under anoxic conditions (Tables 2 and 3).

Azide also stimulates glycolysis, but does not depress the oxidative metabolism to the same extent as cyanide, so that the calculated supply of ATP is maintained at the control value. Under these conditions Cole (1961*a*) showed that sodium transport was markedly stimulated but that the effect could be abolished by dinitrophenol. An essential difference between the effects of cyanide and azide appears to be that for azide an adequate supply of energy is available from oxidative sources, the increased glycolysis in itself being unable to support the higher sodium transport. It remains to be determined whether the metabolic changes are responsible for the total effects of azide or whether changed membrane properties permit the increased transport so long as a minimum energy supply is maintained.

Glucose metabolism in the ciliary body is thus controlled in part at the tricarboxylic acid cycle level and in part by the transport system. Glycolysis, although not essential for sodium transport in terms of energy supply, is maintained at a high rate by interaction with the transporting mechanism, and the lactate produced constitutes a significant part of the secreted aqueous humour.

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The Immunochemistry of *Shigella flexneri* Lipopolysaccharides

A QUANTITATIVE ANALYSIS OF THEIR MONOSACCHARIDE CONSTITUENTS

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1. The lipopolysaccharides of a representative selection of *Shigella flexneri* serotypes all contain the same constituents as *Salmonella* chemotype VII, namely, aldoheptose phosphate, 3-deoxy-2-oxo-octonate, O-phosphorylethanolamine, D-galactose, D-glucose, *N*-acetyl-D-glucosamine and L-rhamnose. 2. The presence of all the *Salmonella* basal sugars in *Sh. flexneri* lipopolysaccharides is consistent with the view that the latter contain a basal structure or core which is similar to the common basal structure of *Salmonella* lipopolysaccharides. 3. Although the *Sh. flexneri* lipopolysaccharides belong to one chemotype, there appear to be quantitative differences in the composition of their O-specific side chains. The repeating units of *Sh. flexneri* serotypes 1a, 2a, 3a, 4a, and variant X contain D-glucose, *N*-acetyl-D-glucosamine and L-rhamnose in the proportions 1:1:2 respectively. The analogous repeating units of serotypes 5a and 6 contain an additional mole of D-glucose and D-galactose respectively and that of variant Y 1 mole of D-glucose less.

There is now much evidence, recently reviewed by Lüderitz, Staub & Westphal (1966), to support the view that the *Salmonella* lipopolysaccharides share a common basal structure or core to which side chains carrying the different O-specific factors are attached. The basal sugars of the core, aldoheptose phosphate, 3-deoxy-2-oxo-octonate, *O*-phosphorylethanolamine, D-galactose, D-glucose and *N*-acetyl-D-glucosamine, are therefore found in all *Salmonella* lipopolysaccharides whereas the 'special' (side chain) sugars associated with O-specificity differ from chemotype to chemotype (Kauffmann, Lüderitz, Stierlin & Westphal, 1960). The more recent demonstration of the same structural sequence in the core of *Escherichia coli* and *Salmonella* lipopolysaccharides (Edstrom & Heath, 1964) and the finding of the *Salmonella* basal sugars in *Arizona* and *E. coli* serotypes (Westphal, Kauffmann, Lüderitz & Stierlin, 1960) indicate the probable presence of a similar basal structure in all Enterobacteriaceae lipopolysaccharides. In the *Sh. flexneri* that form the subject of this paper, the presence of all the basal sugars in a single lipopolysaccharide has yet to be demonstrated but reports of aldoheptose phosphate in *Sh. flexneri* serotype 3 (Slein & Schnell, 1953) and of *O*-phosphorylethanolamine in an unspecified *Sh. flexneri* serotype (Grollman & Osborn, 1964) suggest that the lipopolysaccharides of this genus may also possess a basal structure similar to that of the *Salmonella*.

The present study of eight representative *Sh. flexneri* lipopolysaccharides was undertaken to determine their basal sugar composition and to analyse quantitatively all their sugar constituents including those in the O-specific side chains. Earlier studies (Goebel, Binkley & Perlman, 1945; Slein & Schnell, 1953; Simmons, 1957, 1962) have demonstrated glucose, *N*-acetylglucosamine and rhamnose as the major components of this group of polysaccharides.

MATERIALS AND METHODS

Lipopolysaccharides. The lipopolysaccharides were extracted from acetone-dried cultures of *Shigella flexneri* by the phenol–water method and purified in the ultracentrifuge as described by Kauffmann *et al.* (1960). The N.C.T.C. strain numbers and serotypes of the eight smooth *Sh. flexneri* cultures used are given in Table 1 together with the weights extracted and the yields of lipopolysaccharide obtained.

Chromatography. This was carried out by the descending method on Whatman no. 1 paper with the following solvents: I, butan-1-ol–acetic acid–water (4:1:5, by vol.) (Partridge, 1948); II, butan-1-ol–pyridine–water (6:4:3, by vol.) (Jeanes, Wise & Dimler, 1951); III, ethyl acetate–acetic acid–water (3:1:3, by vol.) (Jermyn & Isherwood, 1949). Alkaline $AgNO_3$ (Trevelyan, Procter & Harrison.

Table 1. *Yield of lipopolysaccharides extracted from some representative* Shigella flexneri *serotypes by the phenol–water method*

Shigella flexneri serotype	N.C.T.C. strain no.	Dry wt. extracted (g.)	Yield of lipopolysaccharide (g.)	Yield of lipopolysaccharide (% dry wt. of bacteria)
1a	3	100	2·29	2·29
2a	4833	85	0·27	0·32
3a	8205	30	0·24	0·80
4a	8206	42	0·81	1·93
5a	8201	50	0·26	0·52
6	8207	30	0·77	2·56
Variant X	2245	36	0·40	1·11
Variant Y	4839	13	0·28	2·15

1950), naphtharesorcinol (Partridge, 1948), the acid–molybdate reagent of Hanes & Isherwood (1949) and ninhydrin (Consden, Gordon & Martin, 1944) were used as identifying reagents both in chromatography and high-voltage electrophoresis.

High-voltage electrophoresis. This was carried out on 2043 bmgl paper (Schleicher & Schüll, Dassel, Germany) according to the method of Kickhöfen & Westphal (1952) with buffer A: pyridine–acetic acid–water (10:4:86, by vol.), pH 5·3. Electrophoresis was run at 3000 v and 120 mA for 1 hr.

Hydrolysis of lipopolysaccharides. Hydrolysis was carried out in sealed glass ampoules, each containing approx. 3·0 mg. of the lipopolysaccharide under study at 1% (w/v) concentration. Samples for amino sugar analysis were hydrolysed for 12 hr. at 100° with 4 N-HCl. Portions with an expected content of 10–50 mμmoles of hexosamine were rapidly dried *in vacuo* to remove the acid. Samples for the analysis of neutral sugars were hydrolysed for 4 hr. at 100° with 1 N-H_2SO_4. The hydrolysate was diluted with 2 vol. of water and neutralized with Amberlite IR-410 (HCO_3^- form) before drying portions *in vacuo* for micro-analysis. Each portion contained 10–100 mμmoles of the sugar under study. For the determination of 3-deoxy-2-oxo-octonate, hydrolysis was for 10 min. at 100° with 0·25 N-H_2SO_4. Negatively charged oligosaccharides, obtained by the elution of high-voltage electrophoretograms, were hydrolysed for 12 hr. at 100° with 2 N-H_2SO_4.

Quantitative microanalysis. Analyses were performed in 7·5 cm. × 1·0 cm. tubes with Carlsberg constriction micro-pipettes (H. E. Pedersen, Copenhagen, Denmark) as follows: heptose by a modified H_2SO_4–cysteine method (Osborn, 1963); 3-deoxy-2-oxo-octonate by the thiobarbituric acid method of Waravdekar & Saslaw (1959); *O*-phosphorylethanolamine by the dinitrofluorobenzene method (Ghuysen & Strominger, 1963); D-galactose both by the galactose dehydrogenase method (Wallenfels & Kurz, 1962) and the galactose oxidase method (Fischer & Zapf, 1964); D-glucose by the glucose oxidase method (Krüger, Lüderitz, Strominger & Westphal, 1962); L-rhamnose both by the H_2SO_4/cysteine reaction (Dische & Shettles, 1951) and the L-rhamnose isomerase method (Krüger *et al.* 1962); free hexosamine by a modified Morgan–Elson reaction after *N*-acetylation (Strominger, Park & Thompson, 1959); D-glucosamine by the D-glucosamine-6-phosphate *N*-acetylase method (Lüderitz, Simmons, Westphal & Strominger, 1964); *N*-acetylhexosamine by a modified Morgan–Elson reaction (Reissig, Strominger & Leloir, 1955), and phosphate by the method of Lowry, Roberts, Leiner, Wu & Farr (1954). All measurements were made in the Unicam SP.500 spectrophotometer with standard microanalytical accessories. The results of the analyses are given in Table 2.

Materials. The enzymes were obtained or prepared as follows: glucose oxidase (C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany); galactose oxidase and horse-radish peroxidase (Seravac Laboratories Ltd., Maidenhead, Berks.); galactose dehydrogenase was prepared by the method of Wallenfels & Kurz (1962) from a culture of *Pseudomonas saccharophila* (a product of Ciba, Basle, Switzerland) kindly given by Dr O. Lüderitz, Freiburg, Germany; D-glucosamine 6-phosphate *N*-acetylase was prepared according to Brown (1962) and L-rhamnose isomerase (also kindly given by Dr O. Lüderitz) was prepared by Krüger *et al.* (1962).

The chemicals were obtained or prepared as follows: D-glycero-L-mannoheptose was a gift from Dr N. K. Richtmyer (National Institutes of Health, Bethesda, Md., U.S.A.) and penta-acetyl methyl ester of 3-deoxy-2-oxo-octonate was a gift from Professor E. C. Heath (Johns Hopkins University, Baltimore, Md., U.S.A.). Free 3-deoxy-2-oxo-octonate was prepared from this derivative by the method of E. C. Heath (personal communication) as follows: 10 mg. of the derivative was dissolved in 1·0 ml. of methanol; 1·0 ml. of 0·2 N-NaOH was added; after mixing and incubating at 37° for 15 min. the mixture was treated with just sufficient Dowex 50 (H^+ form) resin to adjust it to pH 5–6; the resin was removed by filtration and the solution concentrated to dryness under reduced pressure. The sample was dissolved in water to give a molar concentration of 0·01. The pH should be adjusted to 6·5 if necessary.

All other chemicals, reagents and reference sugars were obtained from British Drug Houses Ltd., Poole, Dorset, and were either AnalaR grade or the highest grade obtainable.

RESULTS

The quantitative results given in Table 2 show that the major constituents of the *Sh. flexneri* lipopolysaccharide hydrolysates are glucose, glucosamine and rhamnose. Galactose, heptose

phosphate, 3-deoxy-2-oxo-octonate and *O*-phosphorylethanolamine are present in smaller amounts. From their reactions with the specific enzymes used in the analyses the glucose, galactose and glucosamine are of the D-configuration and the rhamnose is of the L-configuration. The value obtained for the total free hexosamine in each hydrolysate as measured by the method of Strominger *et al.* (1959) was the same as that obtained for glucosamine by the specific *N*-acetylase technique (Lüderitz *et al.* 1964). The total amino sugar content of each lipopolysaccharide is thus accounted for as glucosamine. This conclusion was confirmed by the chromatographic absence of other hexosamines and by the finding of arabinose only when the hydrolysates were treated with ninhydrin–pyridine buffer (Stoffyn & Jeanloz, 1954), which converts amino sugars into their parent pentoses. There is evidence that the free glucosamine in the *Sh. flexneri* lipopolysaccharide hydrolysates is *N*-acetylated in the native polysaccharides (Simmons, 1962). The values given in Table 2 are therefore expressed in terms of *N*-acetyl-D-glucosamine. The presence of galactose, glucose, rhamnose and *O*-phosphorylethanolamine was also confirmed chromatographically. Chromatographic evidence for the presence of heptose and 3-deoxy-2-oxo-octonate in these hydrolysates is unsatisfactory because the former could not be separated from a galactose/glucose mixture and the latter may be confused with oligosaccharides produced during hydrolysis. As heptose phosphate and 3-deoxy-2-oxo-octonate are negatively charged, their presence was convincingly demonstrated by high-voltage electrophoresis as follows: the electrophoretograms of the hydrolysates showed that the bulk of the material was in the neutral fraction but cathodic and anodic fractions were also present, the former being oligosaccharides containing basic amino sugars. Four anodic fractions (electrophoretic mobilities 0·5, 0·6, 0·8 and 1·1 times that of glucose 6-phosphate in buffer A) could be detected in each lipopolysaccharide hydrolysate and these were designated A1 to A4 in order of increasing electrophoretic mobility. A4 was electrophoretically indistinguishable from authentic 3-deoxy-2-oxo-octonate. On elution, it reacted in the thiobarbituric acid reaction (Waravdekar & Saslaw, 1959) to give a chromogen with a spectrum indistinguishable from authentic 3-deoxy-2-oxo-octonate. A1, A2 and A3 all contained phosphorus on spraying with the reagent of Hanes & Isherwood (1949) and with naphtharesorcinol reagent all gave a purple colour indistinguishable from that of glucose 6-phosphate but readily distinguishable from the bright-blue colour of glucuronic acid. A3, when eluted, hydrolysed and chromatographed, gave a single spot indistinguishable from authentic D-glycero-L-mannoheptose in solvents I and II. This experiment does not differentiate between D-glycero-L-mannoheptose and L-glycero-D-mannoheptose but in all Enterobacteriaceae lipopolysaccharides where the configuration has been determined, it has proved to be the latter. Quantitative analysis of the free phosphate in this fraction gave the aldoheptose:phosphate ratio as 1·00:0·71 under the conditions of the experiment. Thus A3 appears to be L-glycero-D-mannoheptose phosphate or its optical enantiomorph. A1 and A2 also contained heptose as evidence by absorption peaks at 510mμ in the sulphuric acid/cysteine reaction (Dische & Shettles, 1951). Absorption peaks at 410mμ in the same reaction indicated the presence of hexose, which on subsequent chromatography proved to be galactose. A1 and A2 therefore appear to be oligosaccharides containing aldoheptose phosphate and galactose. The ratio of the sugars in these anodic oligosaccharides has not yet been established by quantitative analysis. Pentoses, 3,6-dideoxyhexoses and hexuronic acids have not been found by chromatography among the hydrolysis products of these lipopolysaccharides.

The finding of comparable amounts of all the *Salmonella* basal sugars in *Sh. flexneri* lipopolysaccharides is consistent with the view that both genera have a similar type of basal structure or core. If this basal structure contains approximately equivalent amounts of galactose, glucose and *N*-acetylglucosamine as in the *Salmonella* basal structure (Sutherland, Lüderitz & Westphal, 1965), then the bulk of the glucose and *N*-acetylglucosamine of the *Sh. flexneri* lipopolysaccharides lies in the O-specific side chains and the ratio of the sugars in these side chains may be determined as in Table 3.

In the *Sh. flexneri* serotype 6 lipopolysaccharide the galactose ratio is so high that it appears probable that this sugar is also present in the O-specific side chains. Here it has been assumed that the basal galactose accounts for 1% of the total lipopolysaccharide and that equimolar amounts of glucose and *N*-acetylglucosamine are also of basal origin. The analytical results (Table 2) show that the carbohydrate accounted for in serotype 6 is lower than with other serotypes. This lipopolysaccharide may be more resistant to hydrolysis as larger amounts of oligosaccharide were noted when equivalent weights of all lipopolysaccharide hydrolysates were chromatographed.

DISCUSSION

The eight *Shigella flexneri* lipopolysaccharides used in this study all contain the same constituents, namely, aldoheptose phosphate, 3-deoxy-2-oxo-octonate, *O*-phosphorylethanolamine, D-galactose,

Table 2. *Quantitative analysis of the sugar components of* Shigella flexneri *lipopolysaccharides*

Each figure is a mean of eight or twelve determinations on each lipopolysaccharide hydrolysate. The values given are believed to be accurate within the following limits: heptose ± 0·9; 3-deoxy-2-oxo-octonate ± 1·0; *O*-phosphorylethanolamine ± 0·3; galactose ± 1·2; glucose ± 0·7; *N*-acetylglucosamine ± 1·5; rhamnose ± 1·0.

Lipopolysaccharide from *Shigella flexneri* serotype	Percentage of sugar in lipopolysaccharide							Carbohydrate content of lipopolysaccharide* (%)
	Aldoheptose phosphate	3-Deoxy-2-oxo-octonate	*O*-Phosphoryl-ethanolamine	D-Galactose	D-Glucose	*N*-Acetyl-D-glucosamine	L-Rhamnose	
1a	5·6	2·8	3·5	2·0	12·7	17·9	20·8	65·3
2a	5·6	3·0	3·1	2·3	13·1	16·4	22·2	65·7
3a	4·4	3·1	6·1	2·2	11·0	14·5	17·6	58·9
4a	4·9	1·6	2·4	2·2	12·2	15·9	18·4	57·6
5a	4·6	1·5	4·3	2·1	21·9	17·9	22·1	74·4
6	6·0	2·2	8·7	6·5	6·8	8·6	9·7	48·5
Variant X	5·2	1·6	4·9	2·4	12·0	15·4	17·8	59·3
Variant Y	6·0	1·4	3·9	2·4	5·8	17·3	22·2	59·0

* The precise amount of the lipid A component in each lipopolysaccharide remains undetermined. From unpublished observations made during the ether extraction of acetic acid-degraded antigens (Simmons, 1957) it constitutes approx. 30% of the lipopolysaccharide complex.

Table 3. *Molar ratios of sugars in the specific side chains of* Shigella flexneri *lipopolysaccharides*

The 'percentage of sugar in the O-specific side chains' was derived from the data in Table 2, with the following assumptions. All the galactose and equivalent amounts of glucose and *N*-acetylglucosamine are basal in origin. All the rhamnose lies in the O-specific side chains. In serotype 6 lipopolysaccharide where galactose appears to be a side-chain constituent, basal galactose amounts to 1% of the total lipopolysaccharide. In calculating the molar ratios, rhamnose was taken as 2·00.

Lipopolysaccharide from *Shigella flexneri* serotype	Percentage of sugar in the O-specific side chains				Molar ratios of sugars			
	Galactose	Glucose	*N*-Acetylglucosamine	Rhamnose	Galactose	Glucose	*N*-Acetylglucosamine	Rhamnose
1a	0·0	10·7	15·5	20·8	0·00	0·94	1·13	2·00
2a	0·0	10·8	13·6	22·2	0·00	0·89	0·93	2·00
3a	0·0	8·8	11·9	17·6	0·00	0·92	1·02	2·00
4a	0·0	10·0	13·3	18·4	0·00	1·00	1·10	2·00
5a	0·0	19·8	15·4	22·1	0·00	1·63	1·06	2·00
6	5·5	5·8	7·4	9·7	1·02	1·09	1·16	2·00
Variant X	0·0	9·6	12·5	17·8	0·00	0·99	1·06	2·00
Variant Y	0·0	3·4	14·4	22·2	0·00	0·28	1·00	2·00

D-glucose, *N*-acetyl-D-glucosamine and L-rhamnose. They therefore belong to a single chemotype indistinguishable from *Salmonella* chemotype VII (Kauffmann *et al.* 1960). The occurrence of glucose, *N*-acetylglucosamine and rhamnose as the major constituents of these lipopolysaccharides confirms earlier reports by Slein & Schnell (1953) in respect of *Sh. flexneri* type 3 polysaccharide and of Simmons (1957, 1962) in respect of the degraded haptens of all eight serotypes. Reports of small amounts of galactose in the polysaccharides of serotypes 6 (Efimova, 1959), 2a (Sasaki, 1956) and variant Y (Sasaki, 1957), of aldoheptose phosphate in serotype 3 polysaccharide (Slein & Schnell, 1953) and of *O*-phosphorylethanolamine in a *Sh. flexneri* species (Grollman & Osborn, 1964) are also confirmed and extended to the other types used in this study. 3-Deoxy-2-oxo-octonate, which was first isolated from *E. coli* 0111 (Heath & Ghalambor, 1963) and subsequently shown to be a common constituent of *Salmonella* lipopolysaccharides (see review by Lüderitz *et al.* 1966), has now been demonstrated for the first time in the analogous *Sh. flexneri* preparations. The presence of xylose (Sasaki, 1957), of mannose and xylose (Sasaki, 1957) and of uronic acids (Efimova, 1959) in these lipopolysaccharides has not been confirmed with the techniques described above.

The finding of all the *Salmonella* basal sugars in many *E. coli* (see review by Lüderitz *et al.* 1966) and now in *Sh. flexneri* lipopolysaccharides suggests that many Enterobacteriaceae species have a similar lipopolysaccharide core. However, it should be stressed that the presence of a similar core in all Enterobacteriaceae does not necessarily imply that it is structurally identical from genus to genus. Indeed, comparative studies of the lipopolysaccharides from mutants of *Salmonella typhimurium* and *E. coli* 0111 have already demonstrated that, whereas the sugar sequence is the same in both (Edstrom & Heath, 1964), the nature of the linkage in a glucosylgalactose from the *E. coli* lipopolysaccharide is definitely different from that in the analogous glucosylgalactose from *S. minnesota* RII lipopolysaccharide (Sutherland *et al.* 1965).

The O-specific side chains of the *Sh. flexneri* lipopolysaccharides comprise the greater bulk of the total molecule and are probably composed of a number of repeating units analogous to those proposed for *Salmonella* groups B,D,E,G,N and U (Staub & Raynaud, 1964; Robbins & Uchida, 1962; Simmons, Lüderitz & Westphal, 1965*a*,*b*; Lüderitz, Simmons & Westphal, 1965). Although the *Sh. flexneri* lipopolysaccharides all belong to one chemotype, there appear to be quantitative differences in the composition of their O-specific side chains. The results in Table 3 are consistent with the view that the repeating units of serotypes 1a, 2a, 3a, 4a and variant X contain glucose, *N*-acetylglucosamine and rhamnose in the proportions 1:1:2 respectively. The analogous repeating units of serotypes 5a and 6 appear to contain an additional mole of glucose and galactose respectively while that of variant Y has 1 mole of glucose less. It remains to be seen if these quantitative differences are of significance in the immunochemistry of the determinant groups of the different *Sh. flexneri* serotypes.

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Camel Myoglobin

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1. Crystalline myoglobin was prepared from camel heart muscle. 2. A method was developed for the isolation of myoglobin that employs molecular-sieve chromatography. 3. Analytical chromatography of the camel myoglobin on a molecular-sieve column and on two types of ion-exchange columns gave in each case a single elution band, which accounted for better than 98% recovery and showed that the product was free from haemoglobin. 4. The iron content on a dry weight basis was 0·308%. This value corresponds to a molecular weight of 18100. 5. The spectra of acidic ferrimyoglobin, basic ferrimyoglobin and ferrimyoglobin cyanide were measured. 6. The pK_a of the dissociation of the haem-bound water molecule in acidic ferrimyoglobin was 8·53 at 25°. 7. Conclusions are drawn about the charge on the surface of the camel ferrimyoglobin molecule as compared with horse and sperm-whale ferrimyoglobins.

The procedures that have been used by different investigators for the preparation of crystalline myoglobin vary in details, but in principle consist in extraction of myoglobin from the minced muscle with water and subsequent fractionation with ammonium sulphate between 80% and 100% saturation at pH close to 7.

Crystalline myoglobin has been prepared from horse (Theorell, 1932; Roche & Vieil, 1940; Kendrew, 1950; Lawrie, 1951; Kendrew & Trotter, 1954), cow (Roche, Derrien & Vieil, 1942; Rossi-Fanelli, Cavallini & De Marco, 1954; Lewis & Schweigert, 1955), man (Drabkin, 1945; Theorell & de Duve, 1947; Rossi-Fanelli, 1948; Rossi-Fanelli *et al.* 1954; Luginbuhl, 1960), whale (Keilin & Schmid, 1948; Kendrew, Parrish, Marrack & Orlans, 1954; Kendrew & Pauling, 1956; Kendrew & Parrish, 1957; Bradley, 1959), sheep (Beznak, 1948), turtle (Renard, 1953), carp (Hamoir, 1953), seal, penguin, porpoise, California sea lion and dolphin (Kendrew *et al.* 1954), tunny (Huys, 1954; Rossi-Fanelli & Antonini, 1955; Matsuura & Hashimoto, 1955) and molluscs (Rossi-Fanelli, Antonini & Povoledo, 1957).

Haemoglobin is a natural contaminant in the preparation of myoglobin. After the discovery that haemoglobin is insoluble whereas myoglobin is soluble in 3M-phosphate at pH 6·6 (Morgan, 1936), this difference in solubility has been used for the purification of myoglobin. Myoglobin can be separated conveniently from haemoglobin by chromatography on molecular-sieve columns (Awad, Cameron & Kotite, 1963).

Myoglobin has in several instances been shown to be microheterogeneous. For example, subcomponents of myoglobins of the following species have been resolved by ion-exchange chromatography or by electrophoresis: horse (Boardman & Adair, 1956; Åkeson & Theorell, 1960; Reichlin, Hay & Levine, 1963), man (Rossi-Fanelli & Antonini, 1957; Perkoff, Hill, Brown & Tyler, 1962), seal (Rumen, 1959), tunny (Brown, 1961) and sperm whale (Edmundson & Hirs, 1962; Atassi, 1964).

The present work describes the isolation of myoglobin from camel (*Camelus dromedarius*) heart muscle by using molecular-sieve chromatography. The product, ferrimyoglobin, was crystallized from ammonium sulphate solution and some of its physical properties were investigated.

MATERIALS

Whatman CM70 powder (CM-cellulose cation-exchanger) was obtained from W. and R. Balston Ltd., London.

Sephadex G-75 [bead form, fine grade (polydextran molecular sieve of nominal exclusion limit 50000 mol.wt.)], CM-Sephadex C-50 [fine grade (carboxymethyl-polydextran cation-exchanger)] and SE-Sephadex C-50 [fine grade (sulphonylethyl-polydextran cation-exchanger)] were obtained from Pharmacia, Uppsala, Sweden.

Sperm-whale ferrimyoglobin and horse ferrimyoglobin were obtained as salt-free freeze-dried powders from Seravac Laboratories, Maidenhead, Berks.

All other chemicals were of A.R. grade and distilled deionized water was used throughout.

METHODS

Extinction was measured with a Zeiss PMQII spectrophotometer. Silica cells of 1 cm. light-path were used.

Measurement of pH was made with a Radiometer model 4 pH-meter, with glass and calomel electrodes. The instrument was standardized at 25° against phosphate buffer, pH 6·865 (Bates, 1964).

Centrifugation was performed at 4° in a refrigerated MSE centrifuge.

A Cahn electro-microbalance was used for weighing milligram quantities of myoglobin for iron analysis.

All operations in the preparation of myoglobin were carried out in a cold room at 4°.

Molecular-sieve and ion-exchange chromatography were carried out in a cold room at 4° by using standard techniques (Boardman & Partridge, 1955; Porath, 1962).

Iron analysis was performed on 2–8 mg. samples of myoglobin, dried at 110°, by digestion in an $HClO_4$–H_2O_2 medium, reduction of the iron with hydroxylamine hydrochloride and colorimetric assay with *o*-phenanthroline (Cameron, 1965; Hanania, Yeghiayan & Cameron, 1966).

Myoglobin spectra were measured as follows. A stock solution was prepared by dissolving 80 mg. of salt-free freeze-dried myoglobin in 5 ml. of distilled water and filtering. (No significant amount of sediment remained on the filter paper, which indicated the absence of denatured material.) (*a*) Acidic ferrimyoglobin spectrum. A 1·00 ml. portion of the stock solution of myoglobin was diluted to 10·0 ml. with 0·05 M-phosphate buffer, pH 6·2, and the extinction was measured at wavelengths between 450 and 700 mμ against a buffer blank. This solution was diluted further, tenfold, with the same buffer and the extinction was measured at wavelengths between 240 and 450 mμ. (*b*) Basic ferrimyoglobin spectrum. The procedure was the same as that used for acidic ferrimyoglobin, except that the buffer was 0·05 M-borate, pH 10·8. (*c*) Ferrimyoglobin cyanide spectrum. Again the procedure was the same, except for the buffer, which was 0·05 M-phosphate, pH 7·0, containing 0·05% of KCN. For each spectrum, over the range 240–700 mμ, the extinction was measured at about 90 different wavelengths; near the absorption peaks, measurements were made at 2 mμ intervals. The iron content of the stock solution of myoglobin was determined as follows. A 0·300 ml. portion was transferred to a 10 ml. volumetric flask and evaporated to near-dryness on a steam bath. The procedure of Cameron (1965) was then used for the digestion of the myoglobin and the colorimetric assay of iron. The molar extinction coefficients, ϵ ($M^{-1}cm.^{-1}$), were calculated on the basis of an Fe content of 0·308% and 1 Fe atom/mol. of myoglobin (i.e. a molecular weight of 18100) (see the Results section).

RESULTS

Preparation of camel myoglobin. Camel hearts were obtained immediately after slaughter and kept in ice. The muscle was dissected free from fatty tissue and ligaments, washed with tap water, drained and stored frozen at −15° for up to 10 months. Before use the muscle was thawed overnight at 4°. All operations in the preparation of the myoglobin were carried out in a cold room at 4°.

Step (1): extraction. A 3 kg. sample of camel heart muscle was cut into slices, minced in a meat grinder, suspended in 3 l. of ice-cold distilled water and stirred slowly for 24 hr. in the cold. The mixture was squeezed through cheese-cloth, centrifuged at 3000 rev./min. (2400 *g*) for 2 hr. and filtered through Whatman no. 1 fluted filter paper. The pH of the filtrate was 6·4.

Step (2): concentration of myoglobin solution. The filtrate was dialysed extensively against distilled water and twice against 0·01 M-phosphate buffer, pH 6·2, and then filtered to remove a small amount of sediment. The filtrate (3430 ml.) was allowed to percolate through a Whatman CM 70 (CM-cellulose) column (2·5 cm. diam. × 30 cm.) pre-equilibrated with 0·01 M-phosphate buffer, pH 6·2. In this operation the myoglobin and some haemoglobin were adsorbed in a band at the top of the column. After a brief washing with 0·01 M-phosphate buffer, pH 6·2, the band containing myoglobin was eluted from the column with 0·1 M-phosphate buffer, pH 7·3. A cut of the effluent (290 ml.) that contained the myoglobin was taken. About tenfold concentration of the original myoglobin extract was thereby achieved. The E_{410}/E_{280} extinction ratio was 1·92.

Step (3): chromatography on Sephadex G-75. The cut containing myoglobin was applied to a column (5 cm. diam. × 80 cm.) of Sephadex G-75, pre-equilibrated with 0·1 M-phosphate buffer, pH 7·3, and was eluted with the same buffer. The effluent was collected in 15 ml. fractions. The extinction of each fraction was measured at 280 and 410 mμ. When the effluent was too concentrated to be read in the spectrophotometer, a suitable dilution was made, e.g. 0·10 ml. to 5·0 ml. The elution profile is shown in Fig. 1. A cut (315 ml.) was taken by pooling the effluent fractions as indicated in Fig. 1. The E_{410}/E_{280} extinction ratio of the pooled fractions was 4·45.

Step (4): second-cycle chromatography. Steps (2), (3) and again (2) were repeated in succession. A dark-brown solution (67 ml.) of ferrimyoglobin in 0·1 M-phosphate buffer, pH 7·3, was obtained. The E_{410}/E_{280} extinction ratio was 5·08.

Step (5): freeze-drying. The solution of myoglobin was dialysed exhaustively against distilled deionized water and freeze-dried. The fluffy light-brown product (0·79 g.) was stored at 4°.

Crystallization. Suitable conditions for the crystallization of camel myoglobin were determined by setting up 30 small tubes capped tightly and containing 2 ml. of solutions of myoglobin in 75–85% saturated ammonium sulphate buffered with phosphate at pH 6·8. The concentrations of myoglobin, ammonium sulphate and phosphate were varied so that crystallization was attempted under ten different conditions. The tubes were allowed to stand at room temperature (25°) without being disturbed. Crystallization took place within 4 weeks in most of the tubes. The following

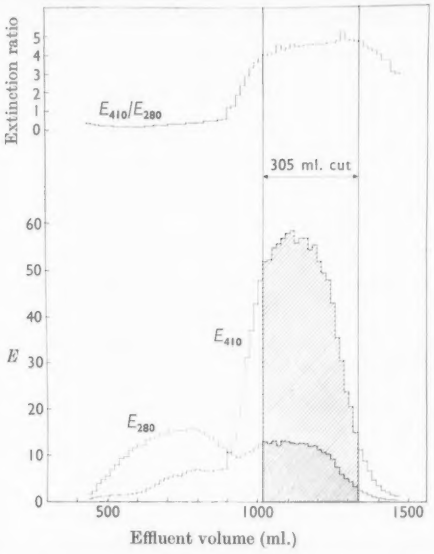

Fig. 1. Elution profile obtained in step (3) in the preparation of camel myoglobin (see the text). A Sephadex G-75 column (5 cm. diam. × 80 cm.) was used, the eluent being 0·1 M-phosphate buffer, pH 7·3.

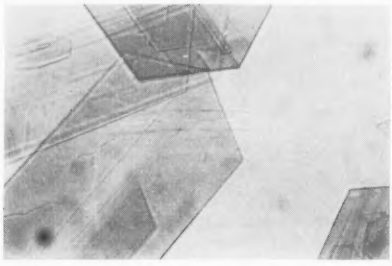

Fig. 2. Camel ferrimyoglobin crystals. Magnification × 50.

procedure was used to crystallize a large amount of myoglobin.

A filtered solution of 0·5 g. of salt-free freeze-dried myoglobin dissolved in 30 ml. of distilled water was mixed with a filtered solution of saturated ammonium sulphate–2 M-phosphate buffer, pH 6·8 (19:1, v/v). A suspension (1 ml.) containing seed crystals was added. The mixture was kept in a glass-stoppered flask at 25° in a thermostatically controlled bath. Crystallization was complete within 4 days.

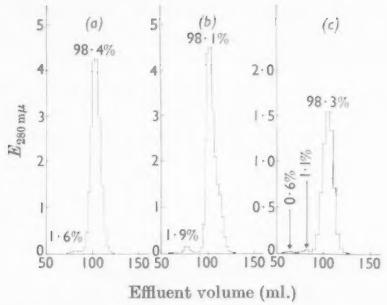

Fig. 3. Chromatographic analysis of camel ferrimyoglobin. (*a*) Molecular-sieve chromatography on a Sephadex G-75 column (2·5 cm. diam. × 34 cm.), the eluent being 0·05 M-phosphate buffer, pH 6·84, and the flow rate 8·4 ml./hr.; the sample contained 40 mg. in 1 ml. (*b*) Ion-exchange chromatography on a CM-Sephadex C-50 column (2·5 cm. diam. × 29 cm.), the eluent being 0·05 M-phosphate buffer, pH 6·85, and the flow rate 9·6 ml./hr.; the sample contained 40 mg. in 1 ml. (*c*) Ion-exchange chromatography on an SE-Sephadex C-50 column (2·5 cm. diam. × 28 cm.), the eluent being 0·05 M-phosphate buffer, pH 6·82, and the flow rate 14 ml./hr.; the sample contained 20 mg. in 1 ml.

The crystals were dark brown, the largest approx. 2 mm. × 0·5 mm. × 0·02 mm. A photograph is shown in Fig. 2.

Analytical chromatography. Samples of camel ferrimyoglobin were analysed by molecular-sieve chromatography on Sephadex G-75, the eluent being 0·05 M-phosphate buffer, pH 6·84, and by ion-exchange chromatography on CM-Sephadex C-50, the eluent being 0·05 M-phosphate buffer, pH 6·85, and on SE-Sephadex C-50, the eluent being 0·05 M-phosphate buffer, pH 6·82. Elution profiles are shown in Fig. 3. In each of the three chromatographic systems, a single elution band was obtained which accounted for at least 98% recovery. Other runs on columns of SE-Sephadex C-50, the eluents being 0·05 M-phosphate buffer at pH 6·40 and at pH 7·42, gave similar elution profiles. These results show that no significant amounts of haemoglobin were present, since both the molecular-sieve and ion-exchange chromatography would have resolved any haemoglobin component in the sample. Comparison of the behaviour of camel ferrimyoglobin with horse and sperm-whale ferrimyoglobins in the same ion-exchange chromatographic system indicates that camel and horse ferrimyoglobins are about equally positively charged at pH 6·85, whereas sperm-whale ferrimyoglobin is considerably more positively charged. This conclusion is borne out by the data in Table 1.

Table 1. *Elution volumes of ferrimyoglobin of different species chromatographed on CM-Sephadex C-50 with* 0·05 M-*phosphate buffer*, pH 6·85

Samples (40 mg.) of myoglobin dissolved in 1·0 ml. of buffer were applied to the column (2·5 cm. diam. × 29 cm.) of CM-Sephadex C-50. The eluent was 0·05 M-phosphate buffer, pH 6·85, the flow rate being 9·6 ml./hr.

	Elution volume (ml.)
Camel ferrimyoglobin	108
Horse ferrimyoglobin (major component*)	129
Sperm-whale ferrimyoglobin (major component*)	520

* Horse and sperm-whale ferrimyoglobins were resolved into one major and several minor components in this chromatographic system (E. S. Awad & L. Kotite, unpublished work). Edmundson & Hirs (1962) have resolved sperm-whale myoglobin into five components on Amberlite IRC-50 columns with 0·34 N-sodium citrate–NaCl buffer, pH 5·88.

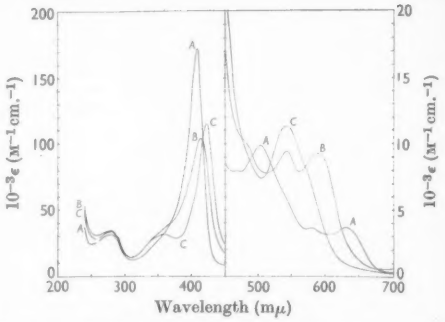

Fig. 4. Spectra of camel myoglobin: *A*, acidic ferrimyoglobin in 0·05 M-phosphate buffer, pH 6·2; *B*, basic ferrimyoglobin in 0·05 M-borate buffer, pH 10·8; *C*, ferrimyoglobin cyanide in 0·05 M-phosphate buffer, pH 7·0, containing 0·05% of KCN. The spectra were measured at room temperature (25°). Molar extinction coefficients are given in Table 2.

The analytical chromatography on CM-Sephadex C-50 and on SE-Sephadex C-50 indicates that the sample of camel ferrimyoglobin was microhomogeneous. Sperm-whale and horse ferrimyoglobins were each resolved into at least four sub-components in the same chromatographic systems.

Iron content and molecular weight. Iron analysis was performed on six samples of myoglobin. The analytical results gave an average value of 0·308 ± 0·002% of iron, which yields a calculated molecular weight of 18 100 on the basis of 1 atom of iron/mol. of myoglobin. The molecular-sieve elution volume, which was obtained in the runs described above, precludes any integral multiple of this molecular weight.

Spectra. The spectra of acidic ferrimyoglobin, basic ferrimyoglobin and ferrimyoglobin cyanide are shown in Fig. 4. Values of the molar extinction coefficients at the maxima, minima and shoulder regions of the spectra are given in Table 2.

Acid dissociation of the haem-bound water molecule. Stock ferrimyoglobin solution (approx. 1 mM) was prepared by dissolving 100 mg. of salt-free freeze-dried myoglobin in 15 ml. of distilled water, adding a few small crystals of sodium nitrite, dialysing twice for 3 hr. against distilled water to remove excess of nitrite and filtering. Of this solution 1·00 ml. was added to 9·00 ml. of buffer of ionic strength 0·05 (phosphate buffer, pH 6·2, glycine buffer, pH 11·0, and ten borate buffers varying in pH between 8·3 amd 10·6). The pH of the mixture was measured with an accuracy of 0·002 pH unit and the extinction was determined at 408 mμ, which is the wavelength of maximum difference in the extinction between the acidic and basic forms of camel ferrimyoglobin.

It was assumed that ferrimyoglobin in the phosphate buffer (pH 6·2) is entirely in the acidic form, i.e. $Fe(H_2O)^+$, and in the glycine buffer (pH 11·0) entirely in the basic form, i.e. FeOH, whereas in the borate buffers there exists a mixture of acidic and basic forms. The degree of dissociation, α, of the haem-bound water molecule, which corresponds to the equilibrium:

$$Fe(H_2O)^+ \rightleftharpoons FeOH + H^+$$

is given by:

$$\alpha = [FeOH]/([Fe(H_2O)^+] + [FeOH])$$

$$= (E_{borate} - E_{glycine})/(E_{phosphate} - E_{glycine})$$

where E is the extinction of the myoglobin solution at 408 mμ and the subscript refers to the buffer in which the extinction was measured. Defining the acid dissociation constant, K_a, by the equation:

$$K_a = [FeOH][H^+]/[Fe(H_2O)^+]$$

then:

$$K_a = \alpha[H^+]/(1-\alpha)$$

where $[H^+]$ is the activity of H^+ ion as measured by the glass electrode. Rearrangement of the last equation gives:

$$\alpha = 1 - \alpha[H^+]/K_a$$

It can be shown that K_a is given by the intercept on the abscissa of a plot of α against $\alpha[H^+]$. A typical plot is shown in Fig. 5. The results of four runs gave an average pK_a value of 8·53 at 25° (average deviation less than ± 0·005).

Table 2. *Spectral characteristic of camel myoglobin*

The spectra are given in Fig. 4. ϵ, Molar extinction coefficient (M^{-1} cm.$^{-1}$); (max.), maximum; (min.), minimum; (sh.), shoulder.

Acidic ferrimyoglobin		Basic ferrimyoglobin		Ferrimyoglobin cyanide	
λ (mμ)	$10^{-3}\,\epsilon$	λ (mμ)	$10^{-3}\,\epsilon$	λ (mμ)	$10^{-3}\,\epsilon$
250 (min.)	24·1	259 (min.)	30·7	257 (min.)	27·7
274 (sh.)	30·8	274 (sh.)	33·6	274 (sh.)	32·7
280 (max.)	31·3	280 (max.)	34·3	280 (max.)	33·3
290 (sh.)	26·6	290 (sh.)	29·3	290 (sh.)	28·1
310 (min.)	12·4	308 (min.)	13·7	308 (min.)	13·7
360 (sh.)	33·0	360 (sh.)	36·2	360 (max.)	31·4
409 (max.)	172·0	414 (max.)	104·0	378 (min.)	28·7
466 (min.)	7·94	480 (sh.)	10·20	423 (max.)	115·0
470 (max.)	7·96	512 (min.)	7·83	505 (min.)	7·45
474 (min.)	7·94	542 (max.)	9·48	542 (max.)	11·32
503 (max.)	9·87	562 (min.)	7·93	570 (sh.)	8·25
573 (min.)	3·55	587 (max.)	9·30		
580 (max.)	3·60	600 (sh.)	8·65		
605 (min.)	3·10	680 (min.)	0·57		
630 (max.)	3·66				
$\epsilon_{410}/\epsilon_{280} = 5{\cdot}49$		$\epsilon_{414}/\epsilon_{280} = 3{\cdot}03$		$\epsilon_{423}/\epsilon_{280} = 3{\cdot}46$	

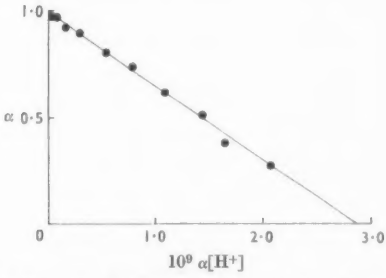

Fig. 5. Example of plot for the determination of pK_a of the haem-bound water molecule in camel acidic ferrimyoglobin (see the text). The intercept on the abscissa gives K_a $2{\cdot}88 \times 10^{-9}$, i.e. p$K_a$ 8·54.

DISCUSSION

The main advantage of the present procedure for the preparation of myoglobin is the ease and thoroughness with which haemoglobin is removed by use of a molecular-sieve column. Other proteins are either not extracted from the muscle with distilled water in step (1) of the preparation, or are not adsorbed on Whatman CM-cellulose in step (2) and are therefore washed out of the column, or are separated out by the molecular-sieve column in step (3). The identity and purity of the product are established by the spectra, the iron analysis and the analytical chromatography.

The molar extinction coefficient of the protein band in camel acidic ferrimyoglobin, ϵ_{280} $31{\cdot}3 \times 10^3\,M^{-1}\,cm.^{-1}$, is very close to the corresponding values for horse acidic ferrimyoglobin (ϵ_{280} $31{\cdot}3 \times 10^3\,M^{-1}\,cm.^{-1}$; G. I. H. Hanania, personal communication) and for sperm-whale acidic ferrimyoglobin (ϵ_{280} $31{\cdot}2 \times 10^3\,M^{-1}\,cm.^{-1}$; Hanania *et al.* 1966). The E_{409}/E_{280} extinction ratio for camel acidic ferrimyoglobin is 5·49, higher than the corresponding values of 5·19 for horse acidic ferrimyoglobin (G. I. H. Hanania, personal communication) and 5·03 for sperm-whale acidic ferrimyoglobin (Hanania *et al.* 1966). This indicates that the electronic distribution in the region of the haem group is different in the myoglobins of these three species.

The pK_a for the ionization of the haem-bound water molecule in camel acidic ferrimyoglobin is 8·53, lower than that for horse acidic ferrimyoglobin (pK_a 8·92 at 20° and I 0·08; George & Hanania, 1952) and that for sperm-whale acidic ferrimyoglobin (pK_a 8·96 at 25° and I 0·05; G. I. H. Hanania, personal communication). The difference of 0·4 pH unit is beyond experimental error. As a check, we have measured the pK_a values for horse acidic ferrimyoglobin and for sperm-whale acidic ferrimyoglobin, using the same buffer solutions as were used in our measurements of the pK_a of camel acidic ferrimyoglobin. For horse acidic ferrimyoglobin we obtained pK_a 8·94 and for sperm-whale acidic ferrimyoglobin pK_a 8·97, in good agreement with the values of Hanania and co-workers. The comparatively low value of the pK_a of the ionization of the water molecule in camel ferrimyoglobin indicates that the local ionic

environment of the haem group in camel ferrimyoglobin, at about pH 8·5, is more positive than in horse and sperm-whale ferrimyoglobins.

In contrast, camel and horse ferrimyoglobins, at about pH 7, have a smaller overall positive surface charge than sperm-whale ferrimyoglobin, as evidenced by the ion-exchange chromatography.

Such species differences are not unexpected, since there is good reason to believe that the amino acid composition and sequence are not identical for myoglobins of different species.

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Metabolism of Cellular Membrane Sulpholipids in the Rat Brain

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1. Metabolism of [^{35}S]sulpholipid was studied in rats of different ages after the injection of [^{35}S]sulphate. 2. The turnover of sulphatide in brain myelin and subcellular fractions was followed for long periods. 3. A small fraction of adult myelin underwent rapid metabolism, the rest being relatively metabolically stable. 4. Fast turnover of brain microsomal sulphatide during development was related to the process of myelination. 5. Turnover of mitochondrial sulphatide was at a lower rate than that calculated for liver mitochondria. 6. The relevance of the results to the metabolism of the whole membrane is discussed.

It has been suggested that the lipids of the central nervous system are almost exclusively associated with membranous structures (Seminario, Hren & Gomez, 1964; Eichberg, Whittaker & Dawson, 1964), for they are located in all subcellular fractions prepared from homogenates with the exception of the soluble fraction, which contains only a very small amount. Probably more than half of the total brain lipid is found in the myelin sheath (Cuzner, Davison & Gregson, 1965*a*,*b*), the rest being largely distributed among mitochondria, microsomes and synaptic endings (Adams, Davison & Gregson, 1963). Since there is evidence to suggest that lipid forms an integral part of the membrane (Gent, Gregson, Gammack & Raper, 1964) it was decided to study the metabolism of a representative membrane lipid for each of the various subcellular fractions of the brain. For this purpose sulphatide (cerebron sulphuric acid) was chosen, for it appears to be a common constituent of biological membranes (Davison & Gregson, 1962). Moreover, labelled sulphate is readily incorporated into brain sulpholipid, where it possesses the same metabolic characteristics as other membrane lipids (Green & Robinson, 1960; Bakke & Cornatzer, 1961; Cuzner *et al.* 1965*a*).

In the present study [^{35}S]sulphate has therefore been utilized for labelling brain membrane structures. Animals of various ages have been injected with [^{35}S]sulphate and the subsequent fate of labelled sulpholipid has been followed in myelin and subcellular fractions, and a comparison has been made of the turnover of each of the various membrane structures of the developing brain with that in the adult rat.

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METHODS

Albino Wistar rats of either sex were used throughout this work. The animals were given carrier-free [^{35}S]sulphate by intraperitoneal injection, except where stated.

Intraventricular injections were performed on adult animals that had previously had the part of the skull just anterior to the frontal–parietal suture and just lateral to the interfrontal suture thinned with a rose-head dental burr. This operation was performed under pentobarbitone anaesthesia at least 24hr. and not more than 48hr. before the injection. For the intraventricular injection animals were anaesthetized and maintained with ether and placed in a head clamp. An Agla micrometer syringe fitted with a 0·5in. 30 B.W.G. needle clamped in a micromanipulator was centred over the exposed region of the thinned cranium. The needle was inserted to a depth of 3mm. below the cranium and 10μl. of carrier-free [^{35}S]sulphate was injected at the rate of 1μl. every 15sec.; 1min. after completion of the injection the needle was slowly withdrawn, the scalp resutured and the animal allowed to revive. The animals normally recovered within 15min. of completing the injection. All results were calculated in terms of an injected dose of 1mc of [^{35}S]sulphate.

Preparation of subcellular fractions. Ether-anaesthetized rats were killed by exsanguination. The brains were quickly removed on to preweighed ice-cold containers. After the weighing, the tissues were suspended in 0·32M-sucrose, containing EDTA (1mM), by using a Potter–Elvehjem type of homogenizer (Aldridge, Emery & Street, 1960; Webster & Smith, 1964). Subcellular fractions were prepared as described by Cuzner *et al.* (1965*b*).

Preparation of extracts. Fractions were extracted with 19vol. of chloroform–methanol (2:1, v/v) and each extract was washed exhaustively by repeated partition against water and 'synthetic upper phase' (Folch, Lees & Sloane-Stanley, 1957) until free of water-soluble radioactivity. Mixtures consisting of large volumes of sucrose solution (e.g. clear supernatant fractions) were extracted by the

method described by Eichberg *et al.* (1964). Where large doses of [^{35}S]sulphate were given by intraperitoneal injection (e.g. adult animals given 11·68 μc) rats under ether anaesthesia were perfused with 0·9% NaCl in 0·01% Na_2SO_4 solution for 5 min. before removing and freezing the brain. Brains from these adult animals and from those injected intracerebrally were homogenized in water (10%, w/v) and 1 ml. portions each treated with 1 ml. of cold 20% (w/v) $HClO_4$. The acid mixture was centrifuged and the clear supernatant obtained was used for acid-soluble ^{35}S estimations. Portions of the aqueous homogenate were extracted with 19 vol. of chloroform–methanol (2:1, v/v), and water-soluble contaminants were removed by partitioning against water (Folch *et al.* 1957) and repeated washing with 'synthetic upper phase' mixtures containing added Na_2SO_4 (0·01%).

Determination of radioactivity. Dried lipid extracts were dissolved in xylene–phosphor mixture and radioactivity was determined in the Nuclear Enterprise scintillation counter NE5503 as described by Davison & Gregson (1962).

Separation and analysis of sulphatide. Samples of washed chloroform–methanol (2:1, v/v) lipid extract were run on thin-layer plates (Davison & Graham-Wolfaard, 1964) developed with chloroform–methanol–water (17:17:1, by vol.). Sulphatide was recovered by transferring silica zones to a column and eluting with 25 ml. of the developing solvent. Sulphatide was best estimated by sphingosine analysis (Lauter & Trams, 1962). This technique was checked by using ^{35}S-labelled brain lipid extracts: 94% of the total radioactivity was recovered in the area corresponding to sulphatide.

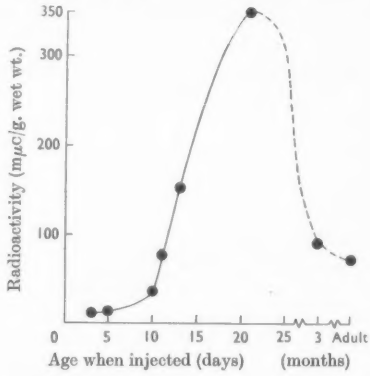

Fig. 1. Sulphate incorporation into whole-brain lipids. Rats of various ages were given carrier-free [^{35}S]sulphate in 0·9% NaCl solution by intraperitoneal injection. Animals were killed after 2 days and the radioactivity of the total washed brain lipid extract was determined. Results shown have been corrected for the brain/body weight ratio for a standard dose of 10 μc of [^{35}S]sulphate/25 g. body wt. per animal.

RESULTS

Previous work has indicated that myelin accounts for more than half the lipid of the central nervous system and that labelled precursors incorporated into such lipid undergo only slow turnover (Davison, 1964; Cuzner *et al.* 1965*a*,*b*). In studying the lipid metabolism of radioactively labelled brain subcellular fractions particular attention has therefore to be paid to separating myelin from each fraction. Thus homogenates of neural tissue in sucrose solution contain myelin fragments of varying size and hence preparations of subcellular fractions by differential centrifugation (e.g. Brody & Bain, 1952) are extensively contaminated (see Laatsch, Kies, Gordon & Alvord, 1962; Gregson, 1965). In the present work myelin has been removed from nuclear and mitochondrial fractions by a single density-gradient centrifugation (Adams *et al.* 1963). The first set of experiments were designed to assess, first, the extent of incorporation of [^{35}S]sulphate into the brain lipids at different ages of rat, correcting the results for brain/body weight ratios, and, secondly, the metabolism of membrane lipid from each of the various cellular fractions of the brain at different stages of development, as judged by uptake of [^{35}S]sulphate *in vivo*.

Incorporation of [^{35}S]*sulphate into sulpholipid of rat brain of different ages.* After interperitoneal injection of [^{35}S]sulphate, the total [^{35}S]sulphate incorporated into whole-brain lipid (Fig. 1) shows a close similarity to that for the incorporation of [1-^{14}C]galactose into rat-brain neutral glycolipids (Burton, Sodd & Brady, 1958) and for the incorporation of [1-^{14}C]glucose into cerebroside galactose from the brain of mice of different ages (Moser & Karnovsky, 1959). Thus maximum amounts of [^{35}S]sulphate are taken up into the brain sulpholipids of the 20-day-old rats, whereas only small amounts are incorporated into brain lipids of rats less than 8 days old. The uptake of [^{35}S]sulphate into the lipids of the adult brain, though more than that found for newborn animals, was considerably less than that for 20-day-old rats. These experiments were repeated on separated subcellular fractions to assess the contribution of each to the metabolism of the whole-brain sulpholipid.

Labelling of myelin and brain subcellular-particle lipids in rats of different ages. Brain homogenates in sucrose solution were prepared from rats of different ages 2 days after injection with [^{35}S]sulphate and were separated into myelin, nuclear, synaptic-ending, mitochondrial, microsomal and supernatant fractions. Labelling of whole-brain homogenate lipids varied with age as indicated in Fig. 1, although the extent of incorporation was different in each of the subcellular fractions (Figs. 2 and 3). Thus the amount taken up into the mitochondria diminished with increasing age of injected rats, whereas the amount incorporated into the nuclear, microsomal,

synaptic-ending and supernatant fractions reached a maximum in animals 15–20 days of age. Radioactivity was extensively incorporated into myelin and this accounted for a high proportion of whole-brain ^{35}S-labelled lipid.

These experiments indicated three broad phases of metabolism, which can be correlated with stages in development of the brain. The first, when there is restricted labelling of brain lipid, coincides with early growth and differentiation before myelination of this part of the central nervous system. The second, represented by rapid sulpholipid synthesis in all fractions (other than mitochondria), parallels the period of rapid myelination (Waelsch, Sperry & Stoyanoff, 1940; Wolman, 1957). The maximal rate of formation of adenosine 3′-phosphate 5′-sulphatophosphate also occurs during this second period (Balasubramanian & Bachhawat, 1961). The final phase is represented by the more steady metabolic state of the adult animal.

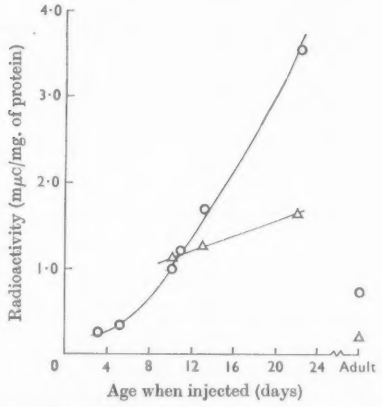

Fig. 2. Sulphate incorporation into the lipids of whole brain and myelin prepared from rats of different ages. Carrier-free [^{35}S]sulphate was injected into groups of animals equivalent to 10 μc/25g. body wt. After 2 days rats were killed and brain homogenates separated as described in the text into myelin and subcellular fractions. Results are expressed as lipid radioactivity/mg. of protein corrected for the brain/body weight ratio; myelin (△) and whole-brain (○) lipid radioactivity is shown. Protein was determined by the method of Murphy & Kies (1960).

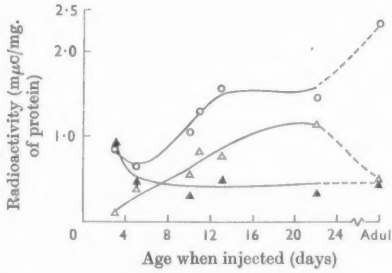

Fig. 3. Sulphate incorporation into the lipids of brain subcellular fractions. Details were as given in Fig. 2. Results for supernatant (△), mitochondria (▲) and microsomes (○) are shown.

Since the amount of [^{35}S]sulphate incorporated after 2 days into each of the brain fractions varies with age, it is necessary to determine whether or not these different uptakes of [^{35}S]sulphate are related to the turnover characteristics of the individual membrane fractions. To elucidate this question metabolism of ^{35}S-labelled lipids of the brain fractions was determined in an extended period after injection of adult, 4-day-old and 12-day-old rats.

Metabolism of sulphatide in brain membrane fractions after the injection of [^{35}S]*sulphate into 4-day-old rats.* Since myelination does not become histologically detectable in the rat until it is 10 days *post partum* it was hoped that injection of labelled precursors into 4-day-old animals would result in labelling of all brain structures other than myelin. Rats of this age were given 25 μc of [^{35}S]sulphate

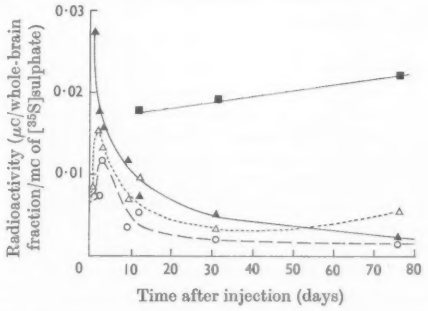

Fig. 4. Turnover of [^{35}S]sulphatide of neonatal rat-brain membrane fractions. The 4-day-old rats were given 25 μc of carrier-free [^{35}S]sulphate by intraperitoneal injection. Animals were killed at various times up to 76 days after injection; in the early experiments brains from two to four animals were pooled to allow separation of about 2g. of nervous tissue. Fractionation and determination of radioactivity were as described in the text. Results shown are radioactivity (μc)/whole-brain fraction corrected to 1 mc of [^{35}S]sulphate injected. Myelin (■) was only separated in animals older than 12 days. Radioactivity in mitochondria, including synaptic endings (▲), microsomes (△) and supernatant (○) are shown. Results for the nuclear fraction are not included.

by intraperitoneal injection and killed at various intervals thereafter. For the first few days after injection two to four rats were killed and the brains pooled for separation into four main fractions: nuclei, mitochondria (including synaptic endings), microsomes and supernatant; myelin, when present, was recovered from the nuclear and mitochondrial pellets. Although there is (Fig. 4) little or no loss of incorporated [^{35}S]sulphate from whole-brain lipid after injection, the distribution of radioactivity in the individual fractions alters considerably so that the increasing labelling of myelin compensates for turnover in other fractions. Moreover, the rate of turnover of labelled lipid is different for each fraction; for example, most [^{35}S]sulphate is taken up into the mitochondrial fraction and subsequent loss of radioactivity is at first rapid with an initial half-life of several days, and thereafter metabolism appears to be much slower. Even after 76 days a trace of ^{35}S-labelled lipid is found in the mitochondrial fraction. Maximum incorporation of [^{35}S]sulphate occurs after 2 days for the supernatant and after 3 days for the microsomal fraction, and again turnover is followed by persistence of radioactivity in each fraction. The nuclear fraction, however, differs from other fractions in that radioactivity, once taken up, remains with little loss. In surviving rats 12 days old or more, myelin was separated from other subcellular fractions. Surprisingly, this fraction contained more than 50% of the whole-brain radioactivity and, moreover, this persisted, with some increase up to the end of the experiment.

Metabolism of sulphatide in brain membrane fractions after the injection of [^{35}S]*sulphate into 12-day-old rats.* In this experiment one litter of six young myelinating rats were injected with 10 μc of carrier-free [^{35}S]sulphate and animals were killed at intervals up to 31 days after injection. Results for brain fractions were recorded graphically and turnover rates computed. During this short experimental period there was only slow turnover of whole-brain lipids (half-life 30–40 days). Distribution of [^{35}S]sulpholipid in the brain showed marked variation for each of the subcellular fractions (see Table 1); only one component was detected in the decay curve for loss of radioactivity from the lipids of all fractions but the experiment was of relatively short duration. [^{35}S]Sulpholipid underwent slow turnover in the nuclear fraction, and no decay (Fig. 5) at all of ^{35}S-labelled myelin was noted (compare with Radin, Martin & Brown, 1957; Davison & Gregson, 1962); the myelin fraction also accounted for the highest proportion of incorporated [^{35}S]sulphate. The results obtained for the mitochondrial and synaptic-ending fractions were very similar to those obtained in the newborn animals (see Fig. 4 and Table 1). Turnover of microsomal and supernatant sulphatide fractions was intermediate between those of the same fractions from neonatal and adult rat brain.

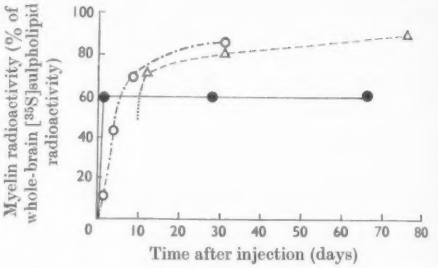

Fig. 5. Incorporation of [^{35}S]sulphate into myelin. Animals were injected when 4 days (△), 12 days (○) or more than 1 year (●) old. Animals were killed at intervals after injection, the brains were removed and fractionated and lipid radioactivity was determined as described in the text. Results shown are total myelin radioactivity as a percentage of whole-brain radioactivity against time.

Incorporation of [^{35}S]*sulphate into the sulpholipids of adult brain.* Fully mature rats of either sex were given carrier-free [^{35}S]sulphate by intraperitoneal injection. At various intervals afterwards animals were anaesthetized, perfused for a few minutes to minimize possible contamination by blood [^{35}S]sulphate and the brains were dissected out. The results showed that equilibrium between brain water-soluble [^{35}S]sulphate and serum was reached in 4hr. Graphical analysis indicated an immediate brain extracellular space of approx. 0·0307 ml./g. fresh wt. with a total sulphate space at equilibrium of 0·0618 ml./g. fresh wt., results that are in accord with those of Woodbury (1958).

Relatively small amounts of [^{35}S]sulphate were, however, incorporated into the brain lipids after intraperitoneal injection; for this reason a reproducible technique for intracerebral injection was developed.

Preliminary experiments with Indian ink indicated that after intracerebral injection there was rapid dissemination of the dose throughout the ventricular system. No damage was noted at the site of the injection, nor were there any discernible abnormalities in treated animals for periods up to a year after injection. It was found that, whereas the rate of incorporation for [^{35}S]sulphate into sulpholipid after intraperitoneal injection was equivalent to the rate of 0·0015 μc/hr./mc of injected [^{35}S]-sulphate, that after intracerebral injection was about 0·15 μc/hr./mc of injected [^{35}S]sulphate. Intracerebral injections were adopted as a standard procedure for adult animals. Besides this greatly enhanced amount of [^{35}S]sulphate incorporation

Table 1. *Comparison of turnover rates obtained from biological decay curves for single fractions from brains of rats of various ages*

The decay constant, K, is calculated as the regression coefficient of the lines $\ln(\text{radioactivity } \mu c) = \ln(a_0 - Kt)$, where t is in days. In the growing animals radioactivity is expressed in terms of the whole brain to allow for the effects of growth. Where the decay of a fraction is biphasic, two constants are calculated, and where necessary the 'fast' phase was corrected for the 'slow' component. The half-life, $t_{\frac{1}{2}}$, in days, was calculated from K. Entry of [^{35}S]sulphate into the slow pool was calculated by extrapolation of the slow-decay curve to $t = 0$, and expressed as a percentage of the total incorporated [^{35}S]sulphate at $t = 0$.

	4-day-old rats		12-day-old rats		Adult rats		
	$-K$	$t_{\frac{1}{2}}$ (days)	$-K$	$t_{\frac{1}{2}}$ (days)	$-K$	$t_{\frac{1}{2}}$ (days)	Entry of [^{35}S]sulphate into slow pool (% of total entry)
Nuclei	0·232	3·0	0·105	6·4	0·263	2·6	—
			—		0·038	18·2	—
Mitochondria		—	0·703	~1·0	1·99	0·35	15·0
					0·018	38·6	
Synaptic endings		—	0·035	19·25	0·232	3·0	—
Combined mitochondria	0·45	1·5	—	—	—	—	—
and synaptic endings	0·055	12·5					
Microsomes	0·065	10·5	0·23	3	1·927	0·36	46·0
					0·077	10·3	
Supernatant	0·134	5·2	0·254	2·7	2·553	0·27	18·0
					0·133	5·2	
Myelin	No detectable decay				0·266	2·7	3·0
					0·005	138	

into brain sulpholipid, the quantity of labelled water-soluble [^{35}S]sulphate fell very rapidly after injection (half-life for acid-soluble sulphate, 1·64 hr.).

Metabolism of [^{35}S]sulpholipid of adult brain membrane fractions. After intracerebral injection [^{35}S]sulphate was incorporated into the lipid of all subcellular fractions in the adult rat brain and the subsequent fate of the [^{35}S]sulpholipid was characteristic for each fraction. Maximal labelling occurred 2–3 hr. after injection in all fractions other than myelin, where peak incorporation was noted 4 hr. after injection (Fig. 5). The myelin fraction accounted for 30–40% and 50–60% of the whole-brain labelled sulpholipid 2 hr. and 4 hr. respectively after injection. The highest specific activity was found in the supernatant fraction (0·296 μc/μmole of sulphatide), whereas the maximal specific activity of the myelin fraction was 0·126 μc/μmole of sulphatide.

The rapid efflux of labelled precursor from the brain of the adult rat has allowed the calculation of turnover rates from data obtained almost immediately after injection as well as from animals from long-term survival experiments. All fractions except synaptic endings showed biphasic decay curves (see also Khan & Wilson, 1965). The supernatant, microsomal and mitochondrial fractions have fast components with half-lives of a few hours (Table 1). By comparison, the fast components for turnover of nuclear and myelin fractions had a half-life of about 2½ days. Results for the metabolism of the slower components in the decay curves for all fractions were comparable with results obtained for the newborn animals.

DISCUSSION

To study the metabolism of the various brain membrane structures sulphatide has been chosen as a representative lipid. Labelling of non-structural sulphatide appears to be of significance only in very short-term experiments, particularly during a period of active lipid biosynthesis. Acid-soluble [^{35}S]sulphate is rapidly excreted from the body (Dziewiatkowski, 1953; Davison & Gregson, 1962), and after intracerebral or intraperitoneal injection negligible amounts are available in the central nervous system after a day. Synthesis of [^{35}S]-sulphatide of high specific activity is therefore restricted to a few hours after injection. In practice, labelling of sulphatide and the subsequent metabolism of [^{35}S]sulpholipid in brain cellular membranes closely resembles that found in similar experiments with ^{14}C- and ^{3}H-labelled precursors (Khan & Wilson, 1965; M. L. Cuzner, A. N. Davison & N. A. Gregson, unpublished work). Thus it seems that in this type of experiment [^{35}S]sulphatide metabolism of each membrane fraction may accurately reflect the metabolism of the whole basic membrane structure.

Metabolism and growth of the myelin sheath.

Inorganic sulphate is incorporated into the whole-brain sulphatide *in vivo* from the first day *post partum* onwards. The proportion of the dose incorporated varies with age (Fig. 1 and Davison & Gregson, 1962), and appears to be related to the overall rate of deposition and to the total amount of myelin in the brain at any age. Thus the incorporation of [^{35}S]sulphate into lipid is minimal in animals aged up to 10 days, when little myelin is present or being formed, and is maximal at about 20 days, coinciding with maximal overall myelination. In the adult rat the extent of incorporation of [^{35}S]sulphate into brain lipid is greater than would be expected if most of the sulphatide were metabolically stable (Davison & Gregson, 1962). Since labelling of myelin sulphatide accounts for most of the incorporated [^{35}S]sulphate the metabolism of this fraction is considered first.

Morphological evidence (Bunge, Bunge & Ris, 1961) indicates that the myelin sheath is derived from the plasma membrane of oligodendroglia in the central nervous system. This membrane fraction is sedimented in the microsomes of brain homogenate. The rapid turnover of microsomal sulphatide and the delayed appearance of labelled myelin found in the present study in the youngest group and to a smaller extent in the 12-day-old rats can then be explained if the myelin sheath is derived from glial-cell membrane. Sulphatide of this newly formed myelin remains for a prolonged period with little turnover (Table 1 and Davison & Gregson, 1962). This is in contrast with the adult rat, where uptake of [^{35}S]sulphate into myelin sulphatide is at a maximum 4hr. after injection and there is a subsequent rapid turnover of most of the labelled sulpholipid. It has been found that the proportion of whole-brain labelled sulpholipid in the myelin of the adult animal stays constant up to 70 days after intracerebral injection (Fig. 5). In the initial period after injection turnover in the myelin parallels that in the subcellular fractions so that the proportions labelled stay about the same. Even after 70 days not all the labelled sulphatide is found in the myelin fraction, although myelin accounts for much more of the persisting radioactivity after injection of the younger rats. There is at present no obvious explanation of these results, which underline the differences in metabolism of labelled myelin in young and adult rats. The rapid attainment of maximum labelling, followed by a relatively rapid rate of turnover, suggests that deposition or possibly the major route of synthesis of myelin membrane differs in the adult from that in the young. The period of rapid decay after the rapid incorporation is followed in turn by a period of slow decay. Such a result suggests that myelin sulphatide in the adult animal can be considered to exist in two metabolic compartments: a small but rapidly exchanging compartment open to a large but slowly exchanging compartment. In a preliminary report of these results this concept was shown diagrammatically (Davison, 1964). From the present results it has been calculated by extrapolation that only 2·9% of the incorporated [^{35}S]sulphate enters the slowly exchanging pool.

On the basis of persistence of isotopic labels in the lipids and proteins in the central nervous system it has been suggested that the myelin sheath is metabolically a relatively inert structure in the adult animal (Davison & Dobbing, 1961). The finding that a part of the [^{35}S]sulphatide incorporated into adult myelin undergoes rapid turnover appears at first to be at variance with this hypothesis. It is thus of great importance to obtain some assessment of the absolute size of this fast pool in the myelin fraction.

From the plot of:

$$\frac{^{35}\text{S incorporation in myelin}}{\int_0^t \text{acid-soluble } ^{35}\text{S} \times dt}$$

against time (t), the initial slope gives an incorporation rate of 0·003075 μc/hr./μc of precursor/g. wet wt. of whole brain equivalent. The values of 2·17 mg. of SO_4^{2-}/100 ml. of rat plasma (Brown & Lewis, 1941) and a brain sulphate space of 0·062 ml./g. wet wt. give a calculated inorganic sulphate content of rat brain of $1{\cdot}347 \times 10^{-6}$ g./g. wet wt. At zero time after intracerebral injection the [^{35}S]sulphate radioactivity is 102 μc/g. wet wt. and the specific activity is 75·82 c/g. of SO_4^{2-}. From this specific activity the incorporation rate is $1{\cdot}1493 \times 10^{-6}$ g. of SO_4^{2-}/sec./g. wet wt. of whole brain equivalent. This incorporation rate can be expressed as $7{\cdot}4817 \times 10^9$ molecules/sec./g. wet wt. of whole brain equivalent. From the decay characteristics of the fast pool of the myelin fraction, the rate of exchange of sulphate in this pool is $0{\cdot}693 \times N/t_{\frac{1}{2}}$ sec.$^{-1}$. This gives an exchange rate for the fast pool of $1{\cdot}6797 \times 10^{18}$ molecules/sec./mole of sulphatide sulphate in the fast pool. Thus the size of this pool is:

$$\frac{\text{observed incorporation rate}}{\text{exchange/mole}}$$

$$= \frac{7{\cdot}4817 \times 10^9}{1{\cdot}6797 \times 10^{18}}$$

$$= 4{\cdot}4544 \times 10^{-9} \text{ moles of sulphatide sulphate}$$

Since the total myelin sulphatide in the rat is $2{\cdot}25 \times 10^{-6}$ moles/g. wet wt. (Cuzner *et al.* 1965*b*) the fast pool as a percentage of the total is only 0·2%.

The small size of the dynamic pool in isolated myelin supports the idea that most of the mature myelin sheath is relatively metabolically stable. The extent of the incorporation and the high

turnover rate into the dynamic pool cannot be explained by the presence of contaminating mitochondria or microsomes, since radioactivity associated with these subcellular fractions is so much less than that of the myelin. Moreover, the calculated size of the dynamic pool of sulphatide precludes it being the complete exterior or interior of the sheaths, since these surfaces would represent some 5–10% of the total myelin membrane. It is possible that the radioactivity of the metabolically active fraction of myelin may be due to sulphatide of a high specific activity or a structure such as the axolemma concentrated in the myelin as prepared. However, a rapidly metabolized polyphosphoinositide component has been described even in a highly purified myelin preparation (Eichberg & Dawson, 1965). Previous work (LeBaron, Hauser & Ruiz, 1962) suggested that the high turnover rate of the phosphoinositides was related to their acting as a 'bridge' between protein and lipid of the membrane structure. Myelin segments, however, are not homogeneous morphologically and it may be that certain regions of the sheath are metabolically active. Thus the paranodal region of fibres in the central nervous system exhibits a region of unspiralling of the myelin lamellae, enclosing oligodendroglial cytoplasm and organelles (Bunge *et al.* 1961; Metuzals, 1964), and may well represent a region of metabolic activity in the central nervous system, as has been suggested for peripheral nerve (Williams & Landon, 1963), and an important site of lipid synthesis. However, although only a small proportion of the myelin is in a dynamic state, this pool still accounts for most of the observed labelling in the adult brain. Thus, although considerable amounts of labelled precursor are incorporated into the central myelin of adult rats (August, Davison & Williams, 1961; Eichberg & Dawson, 1965; Cuzner *et al.* 1965*a*,*b*; Ansell & Spanner, 1965), it does not negate the general validity of the hypothesis that most of the myelin sheath is metabolically stable.

Metabolism of microsomal and mitochondrial sulphatide. Sulphatide is a constituent lipid of brain membrane structures other than myelin (Davison & Gregson, 1962). In general, incorporation of [^{35}S]sulphate into these other structures also varies with age (Figs. 3 and 4; see Abdel-Latif & Abood, 1965, for similar experiments with [^{32}P]-phosphate). Many metabolic and enzyme characteristics of rat brain undergo most rapid change between 10 and 25 days after birth (Flexner, 1955), coinciding with the most rapid structural and functional growth of the brain. Incorporation of [^{35}S]sulphate into the sulphatide of the nuclear, supernatant and synaptic-ending fractions of the brain also changes maximally over this period (Figs. 3 and 4). However, no consistent differences were noted in the metabolism of the brain nuclear and synaptic-ending fractions prepared from animals of different ages, and since the fractions are heterogeneous no comment will be made about their metabolism.

The observed incorporation pattern of [^{35}S]-sulphate into the brain microsomal sulphatide fraction can be related to the period of myelination. Thus the decreased labelling of microsomal sulpholipid 2 days after injection in young compared with adult animals suggests the utilization of glial membrane only during myelination (see above). The pool of rapidly metabolized sulphatide in both microsomal and brain supernatant fractions may represent active biosynthesis of non-membrane sulpholipid (see Table 1), in conformity with current views on the mechanism of sulphatide synthesis (Goldberg, 1961; McKhann, Levy & Ho, 1965).

There is little evidence from the present experiments of any marked difference in the metabolism of brain mitochondrial sulphatide after the first few days *post partum* (see Fig. 4). Although previous results suggested the possibility of a stable component in brain mitochondria (Davison & Gregson, 1962), no such component was found in the present experiments, in which improved techniques were employed. Khan & Wilson (1965) also failed to demonstrate any persistent labelling in the mitochondrial fraction after the administration of ^{3}H-labelled water. The longest half-life for mitochondrial sulphatide turnover in adult animals was approx. 38·6 days (Table 1). If this more stable sulphatide pool is taken to reflect the behaviour of the whole mitochondrial membrane it appears that turnover in the brain is somewhat slower than the half-life of 10·3 days described for liver mitochondria (Fletcher & Sanadi, 1961). Clearly there are two basically different types of membranous structure, myelin representing the metabolically stable type and mitochondria representing the more labile type (Fleischer & Rouser, 1965).

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Isolation and Analysis of the Protoplast Membrane of *Bacillus megaterium*

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1. Optimum conditions were found for the lysis of *Bacillus megaterium* KM by lysozyme. The age of culture, density of suspension and concentration of lysozyme affected the rate of lysis. 2. Protoplast membranes were isolated by centrifugation of lysates and were exhaustively washed. 3. Treatment with chloroform removed some lipid from the membranes, but about half of the total membrane lipid could be extracted only after partial acid hydrolysis. 4. The defatted membranes consisted of protein together with variable amounts of RNA; carbohydrate was almost absent. 5. Lipid accounted for 23% of the weight of the membrane, and included both neutral lipid and phospholipid. In both classes, branched-chain C_{15} acids made up about 80% of the total fatty acid. 6. The phospholipid was a kephalin, and contained small quantities of several amino acids.

The isolation of protoplast membranes has been reported for only a few species of bacteria. One might expect that membranes could be easily obtained from any of the several organisms that are highly susceptible to lysozyme, for example *Micrococcus lysodeikticus*, *Bacillus megaterium* and *Sarcina lutea* (Salton & Pavlik, 1960). In fact, the membranes of *M. lysodeikticus* have been analysed in detail (Gilby, Few & McQuillen, 1958); but those of *B. megaterium* (Weibull & Bergström, 1958) and those of *Sarcina lutea* (Brown, 1961) have received less attention. Some studies have also been made of structures, probably corresponding to the protoplast membrane, which were isolated from organisms that are relatively resistent to lysozyme, such as *Staphylococcus aureus* (Mitchell & Moyle, 1957), *Streptococcus faecalis* (Shockman, Kolb, Bakay, Conover & Toennies, 1963), *Streptococcus* group A (Freimer, 1963) and *Pseudomonas aeruginosa* (Norton, Bulmer & Sokatch, 1963). In summary, these investigations have shown that bacterial membranes are composed primarily of protein and lipid, that carbohydrate is sometimes present and that RNA is a variable component.

The present paper concerns the protoplast membrane of *B. megaterium*. As a necessary preliminary to the analysis of the membrane, optimum conditions were worked out for the lysis of the bacteria with lysozyme. Preparations of membranes were then prepared by centrifugation of cell lysates and were subjected to chemical analysis. A preliminary account of the work has been published (Yudkin, 1962).

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MATERIALS AND METHODS

Maintenance of the organism. *Bacillus megaterium* KM was subcultured at 30° in tubes of Evans peptone (2%, w/v) containing NaCl (0·5%).

Growth conditions. For the preparation of stationary-phase cultures, 5ml. from a 1-day-old culture was added to a 10l. flask containing 4l. of peptone–NaCl medium; when exponential cultures were required a 0·3ml. inoculum was used. The flask was rotated at 120rev./min. at 30°, and the bacteria were harvested by centrifuging, after 16hr. growth unless otherwise specified.

Lysis. The bacteria were resuspended (the density is specified in the text) in a medium containing sodium phosphate buffer, pH 7·0 (0·01M) and $MgCl_2$ (0·01M); 100mμg. of deoxyribonuclease (Worthington Biochemical Corp., Freehold, N.J., U.S.A.) was added/mg. of bacteria. Lysozyme (Armour Pharmaceutical Co., Eastbourne, Sussex) was added at concentrations specified for each experiment, and the suspension was incubated at 37°. Every 5min., a drop of the suspension was examined with the high-power objective of a phase-contrast microscope (magnification 950×). Lysis was judged to be complete when an examination of six independent fields revealed not a single intact organism.

Isolation of membranes. The bacterial lysate was centrifuged at 0° for 20min. at 20000*g*. The pellet of membranes was washed six times with ice-cold 0·1M-NaCl and twice with ice-cold water, and the membranes were finally freeze-dried.

Analytical techniques. Total nitrogen was estimated by

the method of Johnson (1941) and the amino nitrogen of lipid samples by the method of Lea & Rhodes (1955). Total phosphorus was estimated by the method of Fiske & Subbarow (1925), except for the phosphorus of lipid samples, which was determined by the method of Allen (1940). Anthrone (Trevelyan & Harrison, 1952) was used to estimate hexose. For the identification of monosaccharides, material was hydrolysed for 16hr. at 105° with either 2N-HCl or 2N-H_2SO_4; HCl was removed under reduced pressure, and H_2SO_4 with $BaCO_3$. The residues were dissolved in water and chromatographed with butan-1-ol–acetic acid–water (Partridge, 1948) or with ethyl acetate–pyridine–water (Jermyn & Isherwood, 1949). The papers were dried and stained with $AgNO_3$ (Trevelyan, Procter & Harrison, 1950). Hexosamine was estimated by the method of Blix (1948). For the determination of pentose, material was extracted for 1hr. with N-HCl; the extract was centrifuged and treated by the orcinol method (Mejbaum, 1939). A similar acid extract was made in order to detect nucleic acids; HCl was then removed under reduced pressure, and the residue was dissolved in water and applied to Whatman no. 1 paper. The chromatogram was developed with propan-2-ol–HCl (Wyatt, 1951), dried at room temperature and examined in ultraviolet light. Amino acids were detected by chromatographing an acid hydrolysate (McQuillen & Roberts, 1954) and spraying the chromatogram with a solution of ninhydrin (0·5%, w/v) in acetone. Complete amino acid analyses were made by the method of Moore, Spackman & Stein (1958).

Preparation and analysis of lipid. Lipids were kept in solution in chloroform and stored at −10°. Samples were dried in round-bottomed flasks on a rotary evaporator, with the temperature kept below 30°, and were redissolved as soon as possible (in chloroform unless otherwise stated). Extracted lipid was freed of contaminating amino acids by passage through cellulose (Lea & Rhodes, 1953). For 20mg. of lipid a column of 1cm. diam. was packed with Whatman ashless cellulose powder to a height of 20cm.; the lipid was applied in chloroform–methanol (1:1, v/v) and eluted with 30ml. of the same solvent. Neutral lipid and phospholipid were separated by chromatography on silicic acid (A.R. 100 mesh; Mallinckrodt, St Louis, Mo., U.S.A.) as described by Borgström (1952). The silicic acid was washed with chloroform, heated overnight at 105°, washed once more with chloroform and made into a slurry with chloroform. For 20mg. of lipid, a column of 1cm. diam. was packed to a height of 5cm. The lipid was applied in chloroform; neutral lipid was eluted with 20ml. of chloroform, and phospholipid was then eluted with 30ml. of chloroform–methanol (1:3, v/v).

Iodine numbers were estimated by the method of Trappe (1938) and fatty acid ester groups by the method of Rapport & Alonzo (1955). For the detection of nitrogen-containing compounds, samples of lipid were hydrolysed for 15hr. at 105° with 6N-HCl. The acid was removed under reduced pressure and an aqueous extract was applied to a strip of Whatman no. 1 paper. The paper was saturated with pyridine–acetic acid–water (10:0·4:90, by vol.) (pH6·5) and subjected to electrophoresis at 40v/cm. for 1hr. The paper was dried and stained with ninhydrin or with the choline-detecting reagent of Chargaff, Levine & Green (1948). For the determination of ethanolamine, samples of hydrolysed lipid were subjected to electrophoresis, together with known amounts of ethanolamine, at pH2·1 (formic acid, 2·5%, v/v). The paper was dried and stained with a solution of ninhydrin in ethanol (0·5%, v/v); the spots were cut out and eluted, and the colour was read spectrophotometrically (Bode, Hübener, Brückner & Hoeres, 1952).

The fatty acid composition of the lipids was determined by gas–liquid chromatography on a Pye Argon chromatograph. Methyl esters of the fatty acids were prepared by the method of James (1960), and samples were chromatographed on three stationary phases: 8% polyvinyl acetate at 158°, 10% polyethylene glycol adipate at 180° and 10% Apiezon L at 200°. The areas of the peaks corresponding to the separated esters were calculated as half the base multiplied by the height. The proportions of the various esters were found by expressing each area as a percentage of the total.

Solvents. All organic solvents were redistilled. Chloroform was stabilized by the addition of 1% (v/v) of methanol; ether was dried over sodium.

EXPERIMENTAL AND RESULTS

Use of lysozyme. A study of papers that describe the action of lysozyme on *B. megaterium* reveals important variations in the experimental conditions. Suspensions at a density of 10mg./ml. have been converted into protoplasts by lysozyme at 500μg./ml. (Weibull, 1953) or at 100μg./ml. (Weibull, 1957); suspensions of 5mg./ml. have been treated with 300μg. of lysozyme/ml. (Weibull & Bergström, 1958), and suspensions of 30mg./ml. with 1000μg. of lysozyme/ml. (Weibull, 1956). In contrast with these high concentrations of lysozyme used by Weibull, Tomcsik & Baumann-Grace (1956) found that 30μg. of lysozyme/ml. was sufficient for the formation of protoplasts from *B. megaterium*, and Colobert (1961) reported that at concentrations of lysozyme above 300μg./ml. enzymic activity was inhibited.

These differences suggest that neither the density of suspension nor the concentration of lysozyme is of critical importance in the rapid digestion of the cell wall. Moreover, since the authors cited above specify the use of bacteria from stationary cultures, one might conclude that these bacteria are readily susceptible to the action of lysozyme. Neither of these conclusions is in accord with the results described below.

In preliminary experiments, the conditions used were as specified by Weibull & Bergström (1958). When bacteria from stationary cultures were suspended at 5mg./ml. in medium containing 300μg. of lysozyme/ml., lysis of the culture was incomplete even after several hours' incubation. The appearance of a flocculent precipitate suggested that the concentration of lysozyme was too high (cf. Wilcox & Daniel, 1954). The effects of changing the density of the suspension and the concentration of lysozyme were therefore studied; both factors affected the rate of lysis (Table 1).

Even with the best conditions hitherto attained, the incubation had to be prolonged for almost

Table 1. *Effect of suspension density and lysozyme concentration on lysis by lysozyme*

Bacteria were suspended in 0·01 M-sodium phosphate buffer, pH 7, containing $MgCl_2$ (0·01 M) and deoxyribonuclease (100 mμg./mg.).

Suspension density (mg./ml.)	Concn. of lysozyme (μg./mg.)	Concn. of lysozyme (μg./ml.)	Time for lysis (min.)
Stationary-phase culture			
20	50	1000	120
20	20	400	110
20	10	200	180
20	5	100	> 200
10	50	500	190
10	20	200	155
10	10	100	200
10	5	50	> 200
5	50	250	> 200
5	20	100	185
5	10	50	> 200
5	5	25	> 200
Exponential-phase culture			
20	50	1000	> 200
20	20	400	15
20	10	200	20
20	5	100	20
10	50	500	> 200
10	20	200	30
10	10	100	20
10	5	50	20
5	50	250	> 200
5	20	100	50
5	10	50	30
5	5	25	45

2hr. to achieve complete lysis. Microscopic examination showed that it was the long filamentous forms that were most resistant to lysozyme. Since these filaments are characteristic of stationary cultures, and differ in their Gram reaction from the plump rods found in exponential culture, the effect of the age of culture was investigated. Fig. 1 shows that bacteria from the exponential phase are far more susceptible to lysozyme than are those from stationary phase.

The effect of the density of suspension and the concentration of lysozyme was reinvestigated with bacteria from exponential culture, and the conditions that gave the most rapid lysis of stationary cultures were also optimum for exponential cultures (Table 1). In further experiments it was found that washing the bacteria before treatment with lysozyme slightly retarded lysis, and that, although Na^+ is known to stimulate the activity of lysozyme (Wilcox & Daniel, 1954), increasing the concentration of sodium phosphate in the medium from 0·01 M to 0·1 M did not affect the rate of lysis.

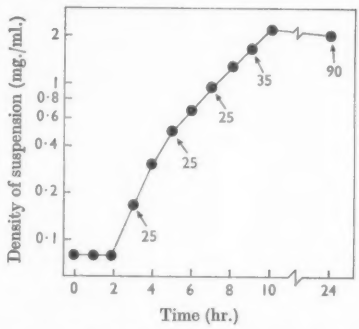

Fig. 1. Effect of age of culture on susceptibility to lysozyme. *B. megaterium* was grown in peptone, and samples were harvested at the points indicated by the arrows. The bacteria were suspended at 20 mg./ml. in 0·01 M-sodium phosphate buffer, pH 7, containing $MgCl_2$ (0·01 M); 100 mμg. of deoxyribonuclease and 20 μg. of lysozyme were added/mg. The numbers next to the arrows show the length of time, in minutes, before complete lysis was attained.

In all the work reported below, the conditions for enzymic digestion were fixed as follows: bacteria harvested from exponential phase were suspended, without washing, at 20 mg./ml. in a medium containing sodium phosphate buffer, pH 7·0 (0·01 M), and magnesium chloride (0·01 M); 400 μg. of lysozyme and 2 μg. of deoxyribonuclease were added/ml., and the suspension was incubated at 37° until lysis was complete.

Analysis of chloroform-extracted membranes. Rods of *B. megaterium* commonly contain granules composed of poly-β-hydroxybutyrate (Lemoigne, 1927). The granules are insoluble in water, and they sediment under the conditions used for centrifuging membranes from a lysate of bacteria. Preparations of protoplast membranes are therefore often contaminated with poly-β-hydroxybutyrate (Storck & Wachsman, 1957).

The crude freeze-dried membranes were freed of poly-β-hydroxybutyrate by extraction with warm chloroform. Five batches of extracted membranes were found to represent 6·5 ± 0·1% by weight of the bacteria from which they had been isolated. The content of nitrogen, phosphorus and ribose is given for the five batches in Table 2. The results suggested that nucleic acid was present, and spots corresponding to the purine bases and pyrimidine nucleotides were detected after paper chromatography of hydrochloric acid extracts. The E_{260}/E_{280} ratio in acid extracts was 1·14. The small amount of anthrone-positive material (Table 2) was identified

Table 2. *Chemical analysis of chloroform-extracted membranes*

The results are given as percentages by weight, and are means of duplicate determinations on each of two separate samples.

Batch	N	P	Ribose	Hexose
1	11·9	0·92	1·40	0·2
2	11·9	0·73	1·28	1·1
3	11·7	0·45	0·75	0·6
4	12·0	0·95	2·49	0·8
5	11·4	1·83	3·54	0·3

Table 3. *Amino acid composition of membrane protein*

The results are given as moles/100 moles.

Cysteic acid	0·1	Methionine	2·3
Aspartic acid	8·8	Isoleucine	6·6
Threonine	5·7	Leucine	8·6
Serine	5·5	Tyrosine	2·1
Glutamic acid	7·2	Phenylalanine	4·6
Proline	5·0	Lysine	6·7
Glycine	7·8	Histidine	1·6
Alanine	8·5	Ammonia	8·6
Valine	7·0	Arginine	3·3

as glucose by chromatography in two chromatographic systems. Hexosamine could not be detected; the sensitivity of the method was sufficient to detect 0·1%. The amino acid composition of the membrane protein is shown in Table 3.

In an attempt to detect teichoic acid (Baddiley, 1962) a sample of the chloroform-extracted membranes was stirred with trichloroacetic acid (10%, w/v) (Armstrong, Baddiley, Buchanan, Carss & Greenberg, 1958), but even when the extraction was continued for 48hr. at room temperature no ethanol-insoluble material was obtained. Teichoic acid was therefore presumed to be absent.

Analysis of the membrane lipid. No further lipid was removed from the chloroform-extracted membranes by a second treatment with chloroform or by extraction with aqueous methanol followed by ether (Reichert, 1944). However, the presence of firmly bound lipid in the membrane was suspected: Weibull (1957) showed that about half of the membrane lipid of *Bacillus* M could be extracted only after treatment with acid. Accordingly, the chloroform-extracted membranes were refluxed for 1hr. with methanol containing 5% (v/v) of concentrated hydrochloric acid (sp.gr. 1·18), the acid methanol was removed under reduced pressure and the residue was extracted three times with ether. Evaporation of the ethereal extracts yielded a lipid fraction. The treatment was repeated and a little more lipid was recovered. The combined ethereal extracts formed fraction MS. The remaining residue was refluxed for 1hr. with 6N-hydrochloric acid, the acid was removed under reduced pressure and three extractions with ether brought out a small quantity of lipid (fraction HS). A further large fraction of membrane lipid was obtained by treating with 5 vol. of ether the chloroform extract of the crude membranes: poly-β-hydroxybutyrate was precipitated by the ether, and the ether-soluble portion of the chloroform extract represented true membrane lipid (fraction CS). The total lipid extracted accounted for 23% of the weight of the membrane.

The membrane lipids were evaporated to dryness, redissolved in chloroform–methanol (1:1, v/v) and passed through cellulose to remove adventitious amino acids (Lea & Rhodes, 1953). They were then separated into neutral lipid and phospholipid. The content of phosphorus and of amino nitrogen in the neutral lipid were both less than 0·1%. The phospholipid contained 3·38% of phosphorus and 1·91% of amino nitrogen. The iodine number of the neutral lipid was 12, and of the phospholipid 5; the fatty acid ester/phosphorus ratio of the phospholipid was 1·7.

A sample of the phospholipid was hydrolysed. Electrophoresis at pH 6·5 showed a pronounced ninhydrin-positive spot running in the position of ethanolamine, and faint spots running with lysine, glutamic acid and the neutral amino acids. Choline was absent. Two-dimensional chromatography revealed ninhydrin-positive spots in the positions of lysine, glutamic acid, glycine, serine, threonine, valine, alanine and leucine or isoleucine; the most intense spot was again in the position of ethanolamine. This last compound was inseparable from ethanolamine when subjected to electrophoresis at pH 2·1 or to chromatography in propan-2-ol–hydrochloric acid (Wyatt, 1951). The ethanolamine/phosphorus ratio in the hydrolysed phospholipid was 0·97.

In preliminary investigations of the fatty acids of the membrane lipid, gas–liquid chromatography on polyvinyl acetate showed the presence of two principal peaks. Both of these (here called *A* and *B*) emerged from the column between methyl tetradecanoate and methyl pentadecanoate. Since on polyvinyl acetate unsaturated compounds run more slowly, and branched-chain compounds more quickly, than the corresponding saturated straight-chain esters, *A* and *B* could have been esters either of unsaturated C_{14} acids or of branched-chain C_{15} acids. However, the low iodine number of the whole lipid tended to rule out unsaturated compounds as major components, and thus both *A* and *B* were probably esters of branched-chain C_{15} acids.

Table 4. *Behaviour on gas–liquid chromatography of standard and unknown esters*

Details of the stationary phases and of the procedure are described in the Materials and Methods section.

Methyl ester of	Relative V_R on Polyvinyl acetate	Apiezon L	Polyethylene glycol adipate
Pentadecanoic acid	0·69	0·64	0·71
Hexadecanoic acid	1·00	1·00	1·00
Octadecanoic acid	2·08	2·44	2·01
12-Methyltridecanoic acid	0·41	0·35	0·42
14-Methylpentadecanoic acid	0·85	0·85	0·85
16-Methylheptadecanoic acid	1·76	2·08	1·70
13-Methyltetradecanoic acid*	0·59	0·54	0·59
12-Methyltetradecanoic acid	0·62	0·57	0·62
A	0·58	0·54	0·59
B	0·62	0·56	0·62

* Calculated by the method of James (1952).

Table 4 gives the relative retention volumes of *A* and *B* and of some standard esters when chromatographed on three stationary phases. Within the limits of experimental error, *B* was indistinguishable from an authentic sample of methyl 12-methyltetradecanoate. An authentic sample of methyl 13-methyltetradecanoate was not obtainable, but its chromatographic behaviour could be predicted from the behaviour of other iso-branched esters by the use of the $-CH_2-$ separation factor described by James (1952). By this means, *A* was indistinguishable from methyl 13-methyltetradecanoate. By similar procedures, minor components of the fatty acid mixture from the membrane lipids were also identified.

13-Methyltetradecanoic acid accounted for 50–55%, and 12-methyltetradecanoic acid for 25–30%, of the total fatty acids. The homologous C_{17} branched-chain acids were present in small quantities (about 2% each). Tetradecanoic acid, pentadecanoic acid, hexadecanoic acid, hexadecenoic acid and octadecanoic acid were also detected.

DISCUSSION

No means are available for showing that a preparation of membranes is entirely free of contamination with other cellular fractions. However, the membranes isolated in the present investigation do appear to be free of cell-wall material, since neither hexosamine nor diaminopimelic acid could be detected in the preparations.

The protoplast membranes of *B. megaterium* consist largely of protein (about 70%) and lipid (about 25%). Teichoic acid and carbohydrate are virtually absent. Membranes of *Bacillus* M are similarly composed principally of protein and lipid, but in that strain variable amounts of glucose (1–10%) also occur (Weibull & Bergström, 1958). Godson, Hunter & Butler (1961) reported that the 'membrane complex' of *B. megaterium* contained over 40% of carbohydrate, but they arrived at this value by difference rather than by direct estimation. On the other hand, membranes of *M. lysodeikticus* were found by the anthrone method to contain 20% of mannose (Gilby *et al.* 1958).

The wide differences in the content of phosphorus and of ribose (Table 2) suggest that variable amounts of RNA are present in the membranes. This variability agrees with contradictory reports about the presence of RNA in protoplast membranes. Gilby *et al.* (1958) failed to detect RNA in membranes of *M. lysodeikticus*, but Gelman, Zhukova & Oparin (1959) found 12·2% of RNA in preparations from the same organism. Membranes of *B. megaterium* have been reported to contain about 3% of RNA (Weibull, 1953), essentially no RNA (Weibull & Bergström, 1958) and 11% of RNA (Vennes & Gerhardt, 1956). The question whether RNA is a genuine constituent of the protoplast membrane of *B. megaterium* has been investigated in detail by Yudkin & Davis (1965).

The difficulty of extracting the membrane lipid has greatly hampered its analysis. Since half of the lipid could be extracted only after acid treatment of the membranes, it is likely that the final fractions obtained include degraded lipid. Nonetheless, some conclusions can be confidently drawn. The phospholipid has a phosphorus/ethanolamine ratio close to 1, and most if not all must therefore be kephalin. This conclusion contrasts with the reports that phospholipids of *Bacillus* M (Weibull, 1957) and of *M. lysodeikticus* (Macfarlane, 1961) are nearly free of nitrogen. The finding of small quantities of several amino acids in the phospholipid recalls similar reports of lipoamino acids in *Penicillium chrysogenum* (Gaby, Hadley & Kaminski, 1957) and in *Pseudomonas aeruginosa*

(Silberman & Gaby, 1961). Hunter & Goodsall (1960, 1961) suggested that amino acids passed through phospholipid in *B. megaterium* before incorporation into protein, and Hendler (1962) proposed a model for protein synthesis that involved incorporation at a lipid–ribonucleoprotein interface. Macfarlane (1962*b*) identified *O*-amino acid esters of phosphatidylglycerol in *Clostridium welchii*, and suggested that these esters might play a role in transport and perhaps in protein synthesis; but Macfarlane (1964*a*,*b*,*c*) has since pointed out that the occurrence of lipoamino acids is not a general phenomenon and that in some Gram-positive organisms their appearance is related to the growth phase. The significance of the discovery of amino acids in the phospholipids described above is still uncertain.

The fatty acid composition of the membrane lipids is as strikingly uniform as that found in other species, with branched-chain C_{15} acids accounting for 80% of the total. The same acids occur in large quantities in *M. lysodeikticus* (Macfarlane, 1961), *Staph. aureus* (Macfarlane, 1962*a*), *Bacillus subtilis* (Saito, 1960) and *Bacillus cereus* (Kates, Kushner & James, 1962). In the cocci the anteiso acid predominates, and in bacilli the iso acid.

The results of this analysis of the protoplast membrane of *B. megaterium* may be contrasted with the composition of the cell wall of the same organism, which has been reported by Ghuysen, Leyh-Bouille & Dericx (1962). The differences once again confirm that the membrane and wall of Gram-positive organism are distinct structures.

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Short Communications

A New Fluorescence Method for Detection and Possible Quantitative Assay of some Catecholamine and Tryptamine Derivatives on Paper

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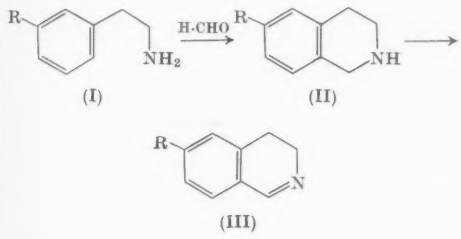
The reaction between catecholamines and formaldehyde in the presence of dry protein films has been discussed by Falck, Hillarp, Thieme & Torp (1962) and its nature has been elucidated by Corrodi & Hillarp (1963, 1964).

These authors have shown that the initial condensation of substituted phenylethylamines (I) with formaldehyde to form tetrahydroisoquinolines (II) takes place readily in solution if an activating group is present in the *meta* position, but that the dehydrogenation of (II) to the fluorescent dihydroisoquinolines (III) by formaldehyde only takes place in a dry film, which can be of protein, as in tissue sections, or of certain amino acids or dipeptides.



We have now used this reaction as the basis for a convenient and sensitive method of detecting catecholamines and their metabolites on paper, and in some cases the reaction products have been eluted and measured quantitatively. In conjunction with other methods, such as ethylenediamine condensation, which depend on different characteristics of the molecule, this should be a valuable tool for identifying these metabolites. Some tryptamines also give fluorescent products.

For test purposes 0·01–10 μg. of the compound was spotted on Whatman no. 1 paper and dried. Optimum conditions for developing the fluorescence were as follows: the papers were sprayed with 5% (w/v) glycine adjusted to pH 3·0 with HCl, and oven-dried at 80° before being suspended in a sealed Kilner jar (3l.) containing paraformaldehyde (20g.) moistened with water (6ml.). The jar was then heated in an oven at 80° for 3hr. and the papers were viewed under u.v. light of wavelength 360 mμ. The fluorescent compounds were eluted from the paper with 0·1N-HCl and their spectral characteristics measured in an Aminco–Bowman spectrophotofluorimeter. These eluates were stable for several days at 0–5°. Dihydroxyphenethylamine (dopamine) and dimethoxyphenethylamine were tested at various loadings and the fluorescence was found to be linear over the range 0·2–4 μg. at least. Table 1 lists the compounds tested and shows the minimum detectable amount, the colour of the spot and in some cases the principal activation and fluorescence maxima of the eluate. Tyramine, dimethoxyphenylacetic acid, histamine, histidine and ergothioneine did not react.

Numerous variations in the conditions were tried before the optimum was arrived at. Glycine was the most convenient 'carrier' reagent: glycylglycine was as effective, but sucrose, starch, phenylalanine and rat-brain homogenate were less effective, as were oxidizing agents, e.g. hydrogen peroxide, periodic acid or ferricyanide. The pH of the glycine treatment was also important, the fluorescence yield

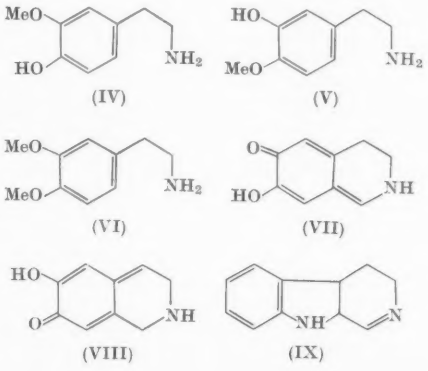


Table 1. *Fluorescence characteristics of catecholamines and other compounds after glycine–formaldehyde treatment.*

Details are given in the text.

Compound	Minimum detectable amount (μg.)	Colour	$\lambda_{max.}$ (mμ) (uncorrected)	
			Activation	Fluorescence
Noradrenaline	0·03	Greenish yellow	430	540
Adrenaline	1	Dull blue–grey		
Dopamine (3,4-dihydroxyphenethylamine)	0·03	Greenish yellow	430	525
Dopa (3,4-dihydroxyphenylalanine)	0·1	Buff–orange	430	530
Metadrenaline	10	Dull olive green		
Normetadrenaline	1	Primrose		
4-Hydroxy-3-methoxyphenethylamine	0·03	Bright yellow	440	520
3-Hydroxy-4-methoxyphenethylamine	0·03	Bright ice blue	360	465
3,4-Dimethoxyphenethylamine	0·03	Bright ice blue	360	470
Mescaline	1	Dull yellow		
Octopamine	10	Dull blue–violet		
α-Methyl-*m*-tyrosine	10	Blue–violet		
4-Hydroxy-3-methoxymandelic acid	10	Apple green		
Tryptamine	0·03	Greenish yellow		
5-Hydroxytryptamine	0·03	Yellow–brown		
Tryptophan	1	Light blue		
Melatonin	1	Yellow–brown		
Bufotenine	1	Dull primrose		
Kynurenine	1	Pale blue–grey		
3-Hydroxykynurenine	1	Buff		
Xanthurenic acid	1	Blue–grey		
Kynurenic acid	1	Light blue		
Anthranilic acid	1	Royal blue		

falling off markedly above pH 3·0. Drying the paper thoroughly before formaldehyde treatment was important, but some moisture in the formaldehyde was necessary though the amount was not critical. The amount of heat required for the formaldehyde reaction was very variable: some compounds, e.g. noradrenaline and dopamine, reacted strongly in 30 min. at 50°, whereas others, e.g. dimethoxyphenethylamine, required much longer. Even at 80° for 3 hr. adrenaline did not react strongly, but further heating offered no advantage.

The differing behaviour of the methoxylated phenethylamines is interesting. Whereas compound (IV) gave a yellow fluorescent product [$\lambda_{max.}$ at 440 mμ (activation) and at 520 mμ (fluorescence) in acid solution] similar to that from dopamine, both compound (V) and compound (VI) gave a blue product [$\lambda_{max.}$ at 360 mμ (activation) and at 470 mμ (fluorescence) in acid solution]. According to Corrodi & Hillarp (1964) dopamine and compound (VI) give products with the same spectrum below pH 5 (the spectrum that we also obtained from compound VI), but above pH 5 differences appear owing to the formation of the quinone (VII) from the dopamine products. Even above pH 5 they did not observe any fluorescent emission higher than 480 mμ, and, since our compounds show their differences in acid solution, it seems likely that our conditions result in further oxidation of the dihydroxy compound with the formation of a more stable quinone form fluorescing at 530 mμ. Further, since compound (V) behaved like compound (VI), where no quinone formation can take place, and dopamine behaved like compound (IV), the quinone must be of the type (VIII) rather than (VII). (Compounds IV and V were obtained from Calbiochem, Lucerne, Switzerland, and their structures have been confirmed by Mr D. Greatbanks by nuclear-magnetic-resonance spectroscopy in alkaline solution: the O^- at position 3 produces a shift in the protons at positions 2 and 6 that does not occur when O^- is in position 4.) Authentic specimens of 6,7-dihydroxy- and 6,7-dimethoxy-3,4-dihydroisoquinolines spotted on paper showed the same blue fluorescence as the product from compound (VI), but after glycine–formaldehyde treatment of the papers the dihydroxy compound altered to give the yellow fluorescence of the product from dopamine, whereas the dimethoxy compound remained unchanged. This is further evidence that some additional reaction has taken place.

The tryptamine fluorescence appeared to be due to a mixture of compounds that were eluted from the paper at different rates, giving rise to variable

spectra in solution. One of these may be the 3,4-dihydro-β-carboline (IX), suggested by Corrodi & Hillarp (1963), which has a yellow fluorescence; the other, and more readily eluted, component has a lower fluorescence maximum and has not been identified.

Falck, B., Hillarp, N.-Å., Thieme, G. & Torp, A. (1962). *J. Histochem. Cytochem.* **10**, 348.

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Corrodi, H. & Hillarp, N.-Å. (1964). *Helv. chim. Acta*, **47**, 911.

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The Rate-Limiting Step in the Oxidation of Palmitate or Palmitoyl-Coenzyme A by Rat-Liver Mitochondria

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It is generally agreed that the rate of β-oxidation of fatty acids in intact mammals is largely controlled by the availability of fatty acids to the tissues concerned (Fritz, 1961), but there is little information or agreement on the rate-limiting steps of fatty acid oxidation in systems supplied with non-limiting concentrations of fatty acids. Reactions catalysed by acyl-CoA synthetase (EC 6.2.1.3; Krebs, 1964; Bode & Klingenberg, 1964) and acyl-CoA dehydrogenase (EC 1.3.2.2; Bunyan & Greenbaum, 1965) have both been described as rate-limiting for fatty acid oxidation.

The low concentration of intermediates of β-oxidation in rat-liver mitochondria oxidizing palmitoylcarnitine (Garland, Shepherd & Yates, 1965) is consistent with organization of the enzymes concerned into a complex, and this possibility is further supported by studies that demonstrate a close relationship between the enzymes of β-oxidation and a barrier or membrane that requires carnitine for acyl transfer (D. W. Yates & P. B. Garland, unpublished work). The activities of enzymes extracted from mitochondria may not represent their normal intramitochondrial activities, and the studies reported below refer to intact mitochondria.

Methods. Preparation of mitochondria and substrates, measurement of mitochondrial oxygen uptake and fluorimetric assay of CoASH with oxoglutarate dehydrogenase (EC 1.2.4.2) (reaction 2) have been described by Garland *et al.* (1965). All mitochondrial incubations were made at 25° in a final volume of 2·1ml. containing KCl (80mM), tris–chloride buffer, pH 7·2 (20mM), $MgCl_2$ (2mM), EDTA (1mM) and mitochondrial protein (1–3mg./ml.). Other additions are described below. The carnitine-palmitoyltransferase activity (EC 2.3.1.–; Norum, 1964) of intact mitochondria was assayed by coupling carnitine-dependent CoASH release from palmitoyl-CoA (reaction 1) with the fluorimetric CoASH assay:

$$\text{Palmitoyl-CoA} + \text{L}(-)\text{-carnitine} \rightleftharpoons \text{palmitoyl-L-carnitine} + \text{CoASH} \quad (1)$$

$$\text{CoASH} + \text{2-oxoglutarate} + \text{NAD} \rightarrow \text{succinyl-CoA} + CO_2 + NADH_2 \quad (2)$$

Further additions to the incubation mixture for this assay were rotenone (0·4μg./ml.), KCN (0·1mM), 2-oxoglutarate (1·0mM), NAD (1·0mM), oxoglutarate dehydrogenase [20μg. (0·06unit)/ml.], palmitoyl-CoA (40μM) and DL-carnitine (2·5mM). Carnitine was added last, and the concentration used was that which gave maximal stimulation of palmitate or palmitoyl-CoA oxidation in respiring mitochondria.

Experimental design. Under suitable conditions (Shepherd, Yates & Garland, 1965) the oxidation of palmitate, palmitoyl-CoA or palmitoylcarnitine by rat-liver mitochondria results in a stoicheiometric conversion into acetoacetate or citrate, and the rate of palmitate or palmitoyl-CoA oxidation (as mμmoles of $C_{16:0}$ oxidized/min./mg. of mitochondrial protein) can be calculated from the observed oxygen uptake rate after correction for the oxidation of endogenous substrates. Under the conditions used, palmitate or palmitoyl-CoA oxidation is absolutely carnitine-dependent, and the correction for oxidation of endogenous substrates is unlikely to include any fatty acid oxidation. The reactions resulting in palmitate oxidation may be

entered at the level of palmitate, palmitoyl-CoA or palmitoylcarnitine, and the relative rates obtained compared with each other and also with the directly measured activities of the steps palmitate → palmitoyl-CoA and palmitoyl-CoA → palmitoylcarnitine.

Results and discussion. Direct assay of the conversion of palmitoyl-CoA into palmitoylcarnitine, at concentrations of palmitoyl-CoA and carnitine that yield maximal rates of oxidation, demonstrates that this conversion proceeds at about 2 mμmoles/min./mg. of mitochondrial protein. This activity is similar to that calculated from the oxidation of palmitoyl-CoA (see Table 1). Direct assay of mitochondrial palmitoyl-CoA synthetase has shown an activity of 5–7 mμmoles/min./mg. of mitochondrial protein (Yates, Shepherd & Garland, 1966), whereas the oxidation of palmitate proceeds at the same rate as that of palmitoyl-CoA. It is concluded that the palmitoyl-CoA-synthetase activity of rat-liver mitochondria is not rate-limiting for palmitate oxidation.

Palmitoylcarnitine is oxidized by rat-liver mitochondria at 3–4 mμmoles/min./mg. of mitochondrial protein, and this rate is independent of whether the end product is citrate or acetoacetate. It is concluded that the conversion of palmitoyl-CoA into palmitoylcarnitine is rate-limiting for both palmitate and palmitoyl-CoA oxidation. Neither the rate of utilization of acetyl-CoA nor electron transport is rate-limiting for palmitoylcarnitine oxidation.

These conclusions may be extrapolated to the intact liver, where at least 90% of carnitine palmitoyltransferase is mitochondrial (Norum, 1965). The increased activity of this rate-limiting enzyme observed in mitochondria from the livers of fat-fed, starved or alloxan-diabetic rats (Norum, 1965) is therefore of particular significance in the regulation of fatty acid oxidation.

Table 1. *Rate of palmitate, palmitoyl-CoA or palmitoylcarnitine utilization by rat-liver mitochondria*

Results are those of a typical experiment. For the oxidation rate studies, additions are to the incubation medium described in the Methods section, which also contained malonate (10 mM), 2,4-dinitrophenol (50 μM) and fluorocitrate (2·5 μM).

Direct enzymic assay

Enzyme	Product	Activity (mμmoles of $C_{16:0}$ converted/min./mg.)
Acyl-CoA synthetase*	Palmitoyl-CoA	5–7
Carnitine palmitoyltransferase	Palmitoylcarnitine	2·4

Oxidation rates

Substrate (with additions)	Final product	Activity (mμmoles of $C_{16:0}$ converted/min./mg.)
Palmitate (40 μM) [+ CoASH (10 μM), oligomycin (5 μg./ml.), ATP (1 mM), DL-carnitine (2·5 mM)]	Acetoacetate	1·7
Palmitoyl-CoA (40 μM) [+ DL-carnitine (2·5 mM)]	Acetoacetate	1·7
Palmitoylcarnitine (40 μM)	Acetoacetate	4·0

* Yates, Shepherd & Garland (1966).

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Asymmetric Transcription of Deoxyribonucleic Acid by Deoxyribonucleic Acid-Dependent Ribonucleic Acid Polymerase of Krebs II Ascites-Tumour Cells

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Early studies of DNA transcription into RNA *in vitro* by using purified bacterial RNA polymerases (EC 2.7.7.6; RNA nucleotidyltransferase) appeared to indicate that the process was symmetrical, i.e. that both strands of the DNA molecule were used as template for the enzymic formation of RNA of complementary base sequence (Geiduschek, Moohr & Weiss, 1962). On the other hand, studies *in vivo* with bacteriophage systems suggested that only one strand of the DNA was used as template for RNA formation (asymmetric transcription) (Hayashi, Hayashi & Spiegelman, 1963; Marmur *et al.* 1963; Tocchini-Valentini *et al.* 1963). Enzymic studies (Geiduschek, Tocchini-Valentini & Sarnat, 1964; Hayashi, Hayashi & Spiegelman, 1964; Green, 1964; Luria, 1965) suggest that there might be some factor in the structure of the enzyme molecule or in the configuration of the template DNA that governs the symmetry or asymmetry of transcription.

This communication presents the results of an investigation into the synthesis of RNA *in vitro* catalysed by the DNA-dependent RNA polymerase associated with the chromatin material from Krebs II mouse ascites-tumour cells. Some of the properties of this enzyme preparation have been described by Eason & Smellie (1965). Such a preparation, it was thought, might contain the template DNA, and any RNA polymerase associated with it, in a configuration close to that occurring *in vivo*. Moreover, no attempt was made to displace histones, or other basic proteins, from the DNA to enhance the activity of the RNA polymerase.

Nuclei were isolated from the Krebs II cells after osmotic disruption of these cells (harvested 6–7 days after implantation) by the method described by Smellie, Keir & Davidson (1959). After collection by centrifugation at 600*g* for 10min., they were washed four times with 10mM-tris–HCl buffer (pH 7·2)–20mM-2-mercaptoethanol (TME buffer) and then ruptured by freezing and thawing three times in 3vol. of TME buffer. The disrupted material was centrifuged at 12000*g* for 10min.; the supernatant fluid was discarded and the sediment was washed twice with further portions of TME buffer to remove any remaining nuclear ribosomes, or other soluble material, and finally suspended in an equal volume of TME buffer. This preparation, which contains the bulk of the cell DNA together with the DNA-dependent RNA-polymerase activity (Eason & Smellie, 1965), was used in this study of the synthesis of RNA *in vitro*. The ^{3}H-labelled RNA was prepared by incubating 0·83ml. of this preparation at 37° for 30min. with 0·16μmole (100μc) of [^{3}H]ATP (Schwarz Bio-Research Inc., Orangeburg, N.Y., U.S.A.), 0·4μmole each of CTP, GTP and UTP, 0·1μmole of tris–HCl buffer, pH 7·2, and 14μmoles of $MnCl_2$ in a final volume of 1ml. After incubation, sodium lauryl sulphate was added (final concn. 0·01%) and the suspension extracted with 80% (w/v) phenol with vigorous shaking for 10min. The resulting emulsion was separated by centrifugation at 10000*g*, and the aqueous phase was removed and re-extracted with phenol and then with ether to remove the phenol traces. After removal of ether with a stream of N_2 the solution was treated with deoxyribonuclease (10μg./ml.) in the presence of $MgCl_2$ (5mM) and bentonite (1mg./ml.). After further phenol extraction as described above the resulting RNA solution was dialysed overnight against 1l. of 15mM-NaCl–1·5mM-sodium citrate, pH 7, and then centrifuged at 20000*g* to remove traces of bentonite. The yield of [^{3}H]RNA from such a preparation ranged from 175 to 500μg. and its specific activity ranged from 181 to 973 trichloroacetic acid-insoluble disintegrations/min./μg. of RNA.

When this ^{3}H-labelled RNA was tested for sensitivity to ribonuclease A under the conditions described by Weissmann, Borst, Burdon, Billiter & Ochoa (1964) up to 6–7% of the labelled RNA was ribonuclease-resistant. Such an observation suggested that at least 92% of the product was single-stranded RNA, while the remaining resistant material might be double-stranded RNA. Further studies using the conditions of Weissmann *et al.* (1964) showed that the ribonuclease-resistance of the ^{3}H-labelled product showed a fairly sharp thermal transition at about 105°, as measured by heating to various temperatures, cooling rapidly and testing with ribonuclease. This is a characteristic of double-stranded RNA rather than an RNA–DNA hybrid (Weissmann *et al.* 1964). It is noteworthy that the

RNA synthesized by a similar enzyme preparation from mouse-liver nuclei was only 1·5% resistant to ribonuclease and this resistance remained unchanged even after heating the RNA to 120°.

To determine whether or not the single-stranded ^{3}H-labelled product synthesized by the Krebs II-cell DNA-dependent RNA polymerase had arisen as a result of the enzymic transcription of both DNA strands, or just one, it was subjected to conditions that might be expected to convert any single strands of RNA having complementary base sequence into a ribonuclease-resistant duplex (i.e. maintaining the RNA solution, 250μg./ml., in 375mM-NaCl–37·5mM-sodium citrate, pH 7·2, for 2hr. at 90° followed by slow cooling). This treatment did not significantly increase the original ribonuclease-resistance of the preparation and indicates that there are not two species of single-stranded RNA synthesized, having complementary base sequence. Further, if the transcription process catalysed by the enzyme preparation is asymmetric, it should be possible to form a hybrid between the ^{3}H-labelled enzyme product and denatured DNA of Krebs II cells, and also to prevent the formation of this hybrid by the addition of a large excess of unlabelled Krebs II-cell RNA. The assumption is made that the RNA isolated from intact Krebs II cells has originated as a result of the transcription *in vivo* from one strand of the cells' DNA. This assumption is supported by the results of hybridization and self-annealing studies with ^{3}H-labelled Krebs II-cell RNA. Such an RNA, isolated after incubating Krebs II cells for 1hr. at 37° in Earle's medium containing 5μc of [^{3}H]-uridine/ml. under the conditions described by Eason, Cline & Smellie (1963), had little resistance to ribonuclease, and, after treatment similar to that already described, which might be expected to anneal RNA strands of complementary base sequence, no significant rise in ribonuclease-resistance could be detected.

Table 1. *Inhibition of hybrid formation between Krebs II-cell DNA and* ^{3}H*-labelled product of Krebs II-cell RNA polymerase by various RNA samples*

DNA was isolated from Krebs II cells by the method of Marmur (1961) and was denatured in water at a concentration of 50μg./ml. by heating at 100° for 10min. followed by rapid cooling. RNA–DNA hybridization was carried out by mixing 12μg. of ^{3}H-labelled enzymically synthesized RNA and 20μg. of denatured Krebs II-cell DNA in a final volume of 1·2ml. of 0·5M-KCl–10mM-tris–HCl buffer, pH 7·2, and maintaining this solution at 67° for 2 hr. and then digesting with 6μg. of ribonuclease A (Worthington Biochemical Corp., Freehold, N.J., U.S.A.) for 30min. at 25°. After this treatment the RNA–DNA hybrids remaining were collected on cellulose nitrate filters by the method of Nygaard & Hall (1963); the filters were dried and then immersed in 5ml. of scintillator fluid [0·5% of 2,5-diphenyloxazole plus 0·03% of 1,4-bis-(5-phenyloxazol-2-yl)benzene in AnalaR toluene)] and assayed for radioactivity in a Packard Tri-Carb liquid-scintillation spectrometer with suitable corrections for quenching.

Origin of unlabelled RNA	Amount of unlabelled RNA added (μg.)	[^{3}H]RNA converted into RNA–DNA hybrid (%)
—	None	8·00
Krebs II ascites-tumour cells	161	3·40
Krebs II ascites-tumour cells	966	0·84*
Hamster cells (BHK21/C13 line)†	360	7·90
Micrococcus lysodeikticus cells	484	7·20

* In this type of experiment no detectable RNA–RNA hybrids were formed.

† Macpherson & Stoker (1962).

The results presented in Table 1 demonstrate that hybrid formation between denatured Krebs II-cell DNA and ^{3}H-labelled product of Krebs II-cell RNA polymerase was considerably inhibited by the addition of a large amount of unlabelled Krebs II-cell RNA. On the other hand, addition of unrelated RNA species had much less effect.

The observation that up to 90% inhibition of hybrid formation could be achieved is significant, since if the RNA synthesized by the RNA polymerase were the result of a symmetrical transcription process then it should only be possible to achieve a 50% inhibition of hybrid formation.

The results of these studies suggest that at least 93–94% of the RNA synthesized by Krebs II-cell DNA-dependent RNA polymerase is single-stranded and arises as a result of the enzymic transcription of only one of the strands of the DNA template.

The significance of the remainder of the product cannot yet be decided. Although it has some of the properties of double-stranded RNA rather than those of an RNA–DNA hybrid, which is formed when single-stranded DNA is used as primer for bacterial RNA polymerases (Warner, Samuels, Abbott & Krakow, 1963; Sinsheimer & Lawrence 1964), the mechanism whereby it might be formed is unknown. One possibility is that, during preparation of the enzyme, the correct enzyme–template relationship is partially destroyed and a small amount of symmetrical transcription is allowed to take place.

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Oxygen Consumption of Isolated Pancreatic Islets of Mice Studied with the Cartesian-Diver Micro-Gasometer

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Studies of the insulin release from the endocrine pancreas and quantitative biochemical analyses of micro-dissected pancreatic islets have significantly contributed to our present knowledge of the function and biochemistry of the pancreatic islet β-cells. However, we have so far only limited information about several important metabolic characteristics of these cells, e.g. the mechanism behind the glucose stimulation of insulin release (cf. Field, 1964). The purpose of the present communication is to demonstrate a new approach to metabolic studies of mammalian β-cells, utilizing Cartesian divers in micro-respirometric analyses of isolated and surviving pancreatic islets.

Methods. Adult female mice with the obese–hyperglycaemic syndrome from a strain originating at the Jackson Memorial Laboratory, Bar Harbor, Maine, U.S.A., were used. The animals were starved for 18hr. before decapitation. The pancreas was then excised and suspended in Krebs–Ringer phosphate buffer, pH 7·4 (Umbreit, Burris & Stauffer, 1964), maintained at 2°. The method used for the subsequent micro-dissection of fresh islets has been described in detail (Hellerström, 1964).

The oxygen consumption of the isolated islets was measured with the aid of Cartesian divers (Linderström-Lang, 1937, 1943; Holter, 1943) with a total volume of 8–12·5 μl. The basic equipment and the handling of the divers mainly conformed to the directions given by Holter (1943, 1961). The silicone-coated divers were either of the 'standard' type, with a bulb between the neck and the tail, or of the cylindrical type. The total gas volume of the charged divers at equilibrium pressure was about 5 μl., i.e. a displacement in the pressure of 1mm. of Brodie solution at 37° corresponded to a change in the gas content of about 0·0004 μl.

When an islet had been isolated from the surrounding tissue, it was washed briefly in fresh Krebs–Ringer phosphate buffer and sucked into a braking pipette (Holter, 1943), together with 0·5 μl. of buffer solution. The medium with the islet was placed as a bottom drop in the bulb of a standard diver, the neck of which was sealed with 0·7 μl. of 1% (w/v) KOH, 0·5 μl. of paraffin oil and an appropriate amount of flotation medium. After transfer to the flotation vessel the diver was allowed to equilibrate for 10min. before the measurements started. When the islet respiration was measured at 30°, the standard divers were filled at room temperature. In experiments performed at 37° the

bottom drop with the islet was also placed as described above, but the diver was then submerged under distilled water in an aluminium thermostat maintained at 37° (Kieler, 1960). When the alkali and oil seals had been introduced, the diver mouth was temporarily closed by a water seal. The diver was transferred to the flotation vessel, where the water seal was replaced by flotation medium and the length of the mouth seal adjusted by means of a fine braking pipette, until the diver equilibrium pressure was a few cm. of Brodie solution below atmospheric pressure (cf. Kieler, 1960).

In a series of experiments designed to study the effect of various exogenous substrates on the islet oxygen uptake at 37°, cylindrical divers were used. They were charged at room temperature with 1 μl. of KOH as bottom drop and a side drop consisting of 0·3 μl. of phosphate buffer containing the substrate to be tested. After submersion of the diver at 37° in the aluminium thermostat, a neck seal consisting of 0·7 μl. of buffer, including the islet, was placed 0·5 mm. above the side drop. The subsequent filling procedure was in accordance with the description above, except that the volume of the oil seal was increased to 1 μl.

Measurements of the oxygen uptake were performed in a water thermostat with a temperature control of $\pm 0{\cdot}004°$. Equilibrium pressures were recorded at intervals of approx. 10 min. and a control diver without an islet was run simultaneously in each experiment. In utilizing cylindrical divers, the endogenous respiration was initially followed for about 1 hr. before the seal with the islet was made to mix with the side drop by the application of a suitable over-pressure (Anfinsen & Claff, 1947). After completion of the measurements the islet was extracted by gently flushing the diver with distilled water from a hand pipette. The islet was subsequently placed on a piece of platinum foil, the weight of which had been determined previously on an ultramicro-balance (Mettler UM7) sensitive to 0·1 μg. The weight of the islet was obtained by reweighing the dried foil.

Results. The dry weights of incubated islets were in the range 1–10 μg., a single islet being incubated in each diver. At both 30° and 37° the respiratory rate was constant for more than 2 hr. At 30° the average ($\pm$S.E.M.) oxygen uptake (Q_{O_2}) in 11 experiments was $3{\cdot}44 \pm 0{\cdot}41$ μl./hr./mg. dry wt., whereas at 37° the Q_{O_2} in 34 experiments had increased to $5{\cdot}41 \pm 0{\cdot}33$ μl./hr./mg. dry wt. A *t*-test showed the difference to be significant at the 1% level.

A striking stimulation of the oxygen uptake was noted when the phosphate buffer was supplemented with 16·7 mM-D-glucose, the Q_{O_2} in 14 experiments at 37° now being $9{\cdot}06 \pm 0{\cdot}55$ μl./hr./mg. dry wt. This value corresponded to an increase of 67%, as compared with that of the glucose-free buffer at the same temperature ($P < 0{\cdot}001$). The effect of glucose on islet respiration was further demonstrated in experiments with cylindrical divers. After the initial measurements of endogenous respiration, glucose was added to the Krebs–Ringer medium in a final concentration of 16·7 mM by mixing with the side drop. As shown in Fig. 1, there was immediately an increased oxygen consumption rate, which remained constant for the rest of the experiment.

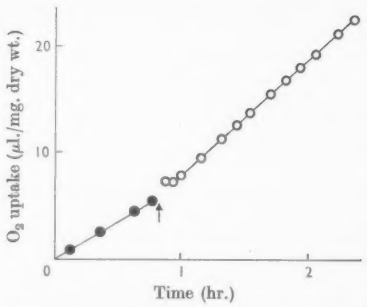

Fig. 1. Oxygen consumption of a pancreatic islet incubated in a cylindrical diver at 37°. The first part of the line (●—●) represents the endogenous respiratory rate in Krebs–Ringer phosphate buffer, and the second part (○—○) shows the respiration after the addition of D-glucose (16·7 mM). The arrow indicates mixing of the buffer seal, including the islet, with the side drop containing glucose. The oxygen uptake increased 58% after the addition of glucose.

Discussion. The present investigation indicates that the technique used for the preparation of fresh mammalian islets preserves the respiratory activity of the islet cells and also provides tissue samples of a size well suited for studies with the Cartesian-diver method. It is notable that the cell damage to isolated islets is kept to a minimum, since each micro-dissected islet is enclosed by an intact connective-tissue capsule (Hellerström, 1964). The increased oxygen consumption induced by a rise in the temperature or the addition of glucose suggests that the gas exchange between the islet cells and the medium was not a limiting factor. Since only small samples of fresh islet tissue can be obtained within a reasonable time, even with special pretreatment of experimental animals (Keen, Sells & Jarrett, 1965) or of the excised pancreas (Moskalewski, 1965), it seems particularly valuable that the diver method should be sufficiently sensitive to measure the respiration of individual mammalian islets.

The endogenous Q_{O_2} value of the present islet material was in the same range as that of a number of other mammalian tissues. It is assumed that this value represents the β-cells, since this cell type

composes more than 90% of the islets of obese–hyperglycaemic mice (Christophe, 1965). The striking stimulation of glucose on β-cell respiration is in line with the observations of Keen *et al.* (1965), who reported a considerably higher oxidation of [1-^{14}C]glucose by isolated rat islets when the glucose concentration of the medium was increased. Friz, Lazarow & Cooperstein (1960) failed, however, to obtain a stimulating effect of glucose on the respiration of islet-tissue slices from toadfish. These results may reflect a significant metabolic difference between pancreatic β-cells in mammals and fish.

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The Enzymic Oxidation of Thiols

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Several observations have been recorded of thiols being able to reduce the coloured products formed by the action of peroxidase and hydrogen peroxide on substrates such as guaiacol and *p*-phenylenediamine, and the early observations have been reviewed by Randall (1946). Glock (1944) observed that thiourea and thiouracil inhibited the action of peroxidase and hydrogen peroxide on pyrogallol. Randall (1946) studied the action of peroxidase and hydrogen peroxide on several thiols and came to the conclusion that thiols do not inhibit peroxidase but are reducing agents acting as substrates for the peroxidase system and competing with other substrates for the available peroxide–peroxidase complex. He also found that thiols can reduce the coloured dyes formed from *p*-aminobenzoic acid and benzidine and thus they show an apparent inhibition of colour production by the peroxidase system.

Neufeld, Green, Latterell & Weintraub (1958) described an enzyme, thiol oxidase, that catalysed the oxidation of thiophenol and sodium diethyldithiocarbamate to the disulphide form, and the reactions catalysed by this enzyme were represented thus:

$$2R\text{–}\overset{\overset{X}{\|}}{C}\text{–}SH + \tfrac{1}{2}O_2 \rightarrow R\text{–}\overset{\overset{X}{\|}}{C}\text{–}S\text{–}S\text{–}\overset{\overset{X}{\|}}{C}\text{–}R + H_2O$$

(where X could be carbon, oxygen, nitrogen or sulphur). The enzyme was purified extensively by Aurbach & Jakoby (1962) and found to contain 0·01% of copper. They reported that the enzyme also catalysed the oxidation of phenols, e.g. catechol and resorcinol. These phenols have also been shown to be oxidized by laccase (*p*-diphenolase) (Benfield, Bocks, Bromley & Brown, 1964).

The oxidation of thiols by a copper oxidase is particularly interesting, since substances such as sodium diethyldithiocarbamate have been used as inhibitors of tyrosinase (*o*-diphenolase), which also contains copper as its prosthetic group (Hallaway, 1959). I have now prepared the thiol oxidase from *Piricularia oryzae* according to the method of Aurbach & Jakoby (1962) and have confirmed their

results with thiophenol, sodium diethyldithiocarbamate, resorcinol and catechol. This enzyme also catalysed the oxidation of other phenols oxidized by laccase. Pyrogallol was oxidized to purpurogallin, 2,6-dimethoxyphenol to 3,3′,5,5′-tetramethoxydiphenoquinone and 2,6-dimethylphenol to 3,3′,5,5′-tetramethyldiphenoquinone. The reactions were carried out under conditions identical with those described in a study of the oxidation of these substrates by laccase from *Polyporus versicolor* and *Rhus vernicifera* and the products were similarly identified (Benfield *et al.* 1964).

Since thiol oxidase from *Piricularia oryzae* resembled laccase in its activity towards phenolic substrates, it was considered likely that laccase would oxidize thiols, e.g. thiophenol and diethyldithiocarbamate, and this has been found to be so with laccase from two sources: a fungal enzyme from *Polyporus versicolor* and a plant laccase from *Rhus vernicifera* were obtained and purified according to methods previously described (Fåhraeus & Lundgren, 1961; Benfield *et al.* 1964). The reactions were carried out on a scale large enough to permit the isolation and identification of the product in each case: 5·0m-moles of the substrate in 100ml. of the appropriate buffer were incubated with 1000units of the enzyme. One unit of laccase caused a change of extinction of 1/min. when 2,6-dimethoxyphenol was oxidized to 3,3′,5,5′-tetramethoxydiphenoquinone, which has $\lambda_{max.}$ at 468mμ. The increase of extinction at this wavelength was used as a measure of laccase activity.

When incubated with thiophenol both enzymes catalysed its oxidation to diphenyl disulphide. The reaction was followed by the spectrophotometric method of Aurbach & Jakoby (1962). A white precipitate formed within 10min. in the incubation mixtures containing active enzyme, but not in the controls that contained boiled enzyme. Reaction mixtures containing *Polyporus versicolor* laccase were buffered at pH4·0 (10mM-sodium acetate) and the *Rhus vernicifera* laccase at pH7·0 (10mM-sodium phosphate). The incubations were carried out at 20° for 120min.; the insoluble precipitate was filtered off and, after recrystallization from ethanol, had an ultraviolet spectrum identical with that of diphenyl disulphide prepared by chemical oxidation of thiophenol with hydrogen peroxide. Each product and a mixture of the two had m.p. 60–61°.

When diethyldithiocarbamate was used as substrate, both enzymes catalysed its oxidation to tetraethylthiuram disulphide. However, as diethyldithiocarbamate is unstable at pH values below 6·0, the reactions were carried out at pH6·0 with the *Polyporus versicolor* enzyme and at pH7·0 with the *Rhus vernicifera* enzyme; with each enzyme 10mM-sodium phosphate buffer was used. Here, too, a cloudy white precipitate formed after 10min. incubation at 20° in reaction mixtures containing active enzyme. After 180min. incubation, the insoluble white product was filtered off and recrystallized from ethanol. The product was identical with tetraethylthiuram disulphide prepared by oxidation of sodium diethyldithiocarbamate with potassium ferricyanide. The ultraviolet spectra of the two products were identical, and each product and a mixture of the two had m.p. 69–70°. It was concluded that both the aromatic compound thiophenol and sodium diethyldithiocarbamate, a non-aromatic compound containing thiol groups, were substrates of laccase from *Polyporus versicolor* and *Rhus vernicifera* or that a thiol oxidase was closely associated with both these enzymes and not separated by the procedures used for isolation.

The present results appear to support the findings of Aurbach & Jakoby (1962) for the *Piricularia oryzae* thiol oxidase, where the oxidation of thiols and phenols was catalysed by the same oxidase. They also reported a lack of activity towards thiophenol by a plant tyrosinase (*o*-diphenolase). This may be connected with the state of oxidation of copper in tyrosinase. Kertész (1957) has shown that copper is always present in the univalent state in purified mushroom tyrosinase, and similar results have been obtained for purified *Neurospora* tyrosinase (Fling, Horowitz & Heinnemann, 1963). Univalent copper forms very stable complexes with thiols, which could be very effective inhibitors of tyrosinase activity.

However, both *Polyporus versicolor* laccase and *Rhus vernicifera* laccase have been shown to contain copper in the bivalent state. Nakamura (1960), using electron-spin-resonance spectroscopy, has shown that all the copper in *Rhus vernicifera* laccase is present as Cu^{2+}, and by the same technique Malström, Mosbach & Vänngård (1959) have shown that copper exists partly as Cu^{+} and partly as Cu^{2+} and that a valency change (Cu^{2+} to Cu^{+}) occurs during the oxidation of the substrates. Hence the mechanism of oxidation catalysed by the blue laccase enzymes containing Cu^{2+} may be very different from that of the colourless tyrosinase enzymes containing Cu^{+}; any inhibition by thiols, of the oxidation of phenols or other substrates by laccase, may be complicated if thiols also act as substrates and as reducing agents for the coloured quinones produced during the oxidation of phenols and amines.

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Zinc as a Cofactor for Cephalosporinase from *Bacillus cereus* 569

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Crude preparations of β-lactamase from *Bacillus cereus* 569 show a selective loss of cephalosporinase activity on purification (Abraham & Newton, 1956) and during prolonged action on substrate (Sabath & Abraham, 1965). They also show a much greater loss of penicillinase (EC 3.5.2.6) activity than cephalosporinase activity on keeping at 60° (Crompton, Jago, Crawford, Newton & Abraham, 1962). During attempts to purify the crude preparation cephalosporinase activity was repeatedly lost although penicillinase activity was retained. It was therefore decided to investigate the possibility that a cofactor required only for cephalosporinase activity was removed during the purification procedures used.

Solutions of the crude enzyme from *B. cereus* 569 were prepared as described by Crompton *et al.* (1962) with cephalosporin C_A (pyridine) (10μg./ml.) as inducer and an induction time of 3hr. β-Lactamase activities were measured manometrically (Henry & Housewright, 1947; Pollock, 1952) and related to μl. of CO_2/ml. of enzyme/hr., normally with substrate (cephalosporin C or benzylpenicillin) at a concentration of 2mg./ml.

Table 1 shows that some loss of cephalosporinase activity occurred when the crude enzyme was dialysed against running tap water, but that when EDTA (disodium salt) was added before dialysis (final concn. 0·27mM) the selective loss of cephalosporinase was much more striking. A similar extensive loss of cephalosporinase activity was observed when measurements were made with enzyme that had been treated with EDTA (at concentrations down to 2·7μM) but not dialysed.

Cephalosporinase activity was restored to solutions of crude enzyme that had been subjected to treatment with EDTA and dialysis by the addition of $ZnSO_4$. When the latter was added in optimum amount (final concn. 1mM) the cephalosporinase activity rose to 4–5 times that of the original crude enzyme (Table 1). Addition of Zn^{2+} to the original crude enzyme, or to the dialysed enzyme, also produced a similar increase in cephalosporinase activity.

Several other metal-binding substances decreased the cephalosporinase activity of the crude enzyme to a relatively low level. After the addition of 1,10-phenanthroline (300μM) the cephalosporinase activity was less than 10% of its original value and the penicillinase activity 95%. With 240μM-quinalizarin (tetrahydroxyanthraquinone) the corresponding value for cephalosporinase activity was also less than 10%. After H_2S had been bubbled gently through the solution for about 7min. the values for cephalosporinase and penicillinase activities were less than 10% and 90% respectively. No significant loss of cephalosporinase activity was observed after treatment of the crude enzyme with ammonium aurin tricarboxylate (140μM), titan yellow (160μM), sodium azide (10mM) or 8-hydroxyquinoline (1mM). The use of 8-hydroxyquinoline (1mM) to terminate enzyme induction (Crompton *et al.* 1962) did not significantly affect the cephalosporinase activity of the crude enzyme obtained.

The logarithms of the equilibrium formation

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Table 1. *Effect of EDTA and* Zn^{2+} *on the cephalosporinase and penicillinase activities of crude β-lactamase from* B. cereus *569*

The cephalosporinase activity of the original crude enzyme (μl. of CO_2/ml.of enzyme/hr.) was about 4% of the penicillinase activity. Dialysis or measurement of activity was begun between 3 and 15 min. after the addition of EDTA. — Signifies that the treatment referred to was omitted.

Treatment of crude enzyme			Activity (% of that of original crude enzyme)	
(1) EDTA (0·27 mM) added	(2) Dialysis (15–18 hr.) after (1)	(3) Final concn. of added $ZnSO_4$ (mM) after (1) and/or (2)	Cephalosporinase	Penicillinase
—	—	—	100	100
—	+	—	75	100
—	+	1·0	530	135†
+	—	—	3	89
+	+	—	< 12*	93
+	+	10·0	191	—
+	+	1·0	443	120†
+	+	0·1	151	—
—	—	1·0	525	150†
—	—	0·01	157	—

* A series of four experiments gave three values of < 8% and one of 11%; but in two other experiments the values were 25 and 30% respectively.

† Some, at least, of the apparent increase in activity in these cases may be attributed to the fact that benzylpenicillin was hydrolysed at a significant rate in the absence of enzyme when Zn^{2+} was present.

constants, K, for 1:1 chelates of EDTA and 1,10-phenanthroline with zinc are 16·1 and 6·43 respectively (Martell & Calvin, 1952). It is therefore not surprising that the former ligand was effective in lower concentrations than the latter in removing cephalosporinase activity. However, the failure of 8-hydroxyquinoline ($\log K$ 10·91) to act as an inhibitor suggests that the effect of a ligand also depends on its ability to approach a zinc-binding site in the enzyme.

In concentrations from 100 to 1 mM a variety of metal salts other than $ZnSO_4$ did not restore more than a small fraction of the cephalosporinase activity of crude enzyme that had been subjected to treatment with EDTA and dialysis. The metal ions used were Ca^{2+}, Ba^{2+} and Cd^{2+} (added as their chlorides), Mg^{2+} and Mn^{2+} (added as their sulphates) and Co^{2+} (added as its acetate). The addition of $CuSO_4$ or $FeSO_4.(NH_4)_2SO_4$ (1 to 0·01 mM) to the original crude enzyme produced no significant change in cephalosporinase activity. At higher concentrations the effect of these metal ions was inhibitory.

Cephalosporin C acted as a competitive inhibitor of the penicillinase activity of the crude enzyme preparation from *B. cereus* 569 and also of the preparation whose cephalosporinase activity had been largely removed by treatment with EDTA and dialysis. Values of K_i/K_m with benzylpenicillin as substrate and cephalosporin C as inhibitor were obtained from data plotted in the manner of Hunter & Downs (1945). The value of K_i/K_m, i.e. 4, with the former preparation did not differ significantly from that with the latter.

The present results suggest that without zinc as a cofactor the crude enzyme from *B. cereus* 569 shows little, if any, of its potential cephalosporinase activity. In contrast, it has been concluded that there is no indication of a cofactor requirement for the penicillinase from this organism (Pollock, 1960). Whether activation by zinc is peculiar to cephalosporinase from *B. cereus* is uncertain, but it does not appear to be a general characteristic of cephalosporinases, since the crude enzyme from *Pseudomonas pyocyanea* (Sabath, Jago & Abraham, 1965) was not inhibited by EDTA or by H_2S. Moreover, changes other than, or additional to, loss of zinc may result in a selective loss of cephalosporinase activity of crude enzyme from *B. cereus*. A preliminary attempt to increase the relatively minute amount of cephalosporinase activity in a highly purified preparation of penicillinase from *B. cereus* 569/H (kindly provided by Professor M. R. Pollock) by the addition of Zn^{2+} (100 to 0·01 mM) was unsuccessful.

The finding that cephalosporin C was a competitive inhibitor of penicillinase in the crude enzyme from *B. cereus* 569 both before and after the cephalosporinase activity of the latter had been lost on treatment with EDTA, as well as an inhibitor of

a purified penicillinase from *B. cereus* 569 that had retained very little cephalosporinase activity (Abraham & Newton, 1956), indicates that cephalosporin C can form a complex with a β-lactamase from this organism in the absence of Zn^{2+}, although Zn^{2+} is necessary for enzymic hydrolysis of cephalosporin C to occur at a significant rate. This situation could be analogous to that with carboxypeptidase, whose zinc-free apoenzyme can form complexes with certain substrates but is unable to catalyse their hydrolysis (Coleman & Vallee, 1962).

Whether Zn^{2+} is incorporated into the active centre of the cephalosporinase or whether a change in conformation at the active centre results from a reaction with Zn^{2+} at another part of the molecule is not revealed by the present data. The question arises whether the cephalosporinase and penicillinase activities are associated with a single protein whose conformation is changed by complex-formation with zinc. Purification of the crude enzyme from *B. cereus* 569 under conditions such that its cephalosporinase activity is retained may be necessary before this question can receive a satisfactory answer.

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Studies on the Biosynthesis of the Corrin Ring of Vitamin B_{12}

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The similarity in structure between the corrin ring of vitamin B_{12} and the porphyrin nucleus of haem has stimulated numerous workers to examine the similarities in the biosynthetic pathways (Bukin & Pronyakova, 1960; Shaposhnikov & Finogenova, 1963; Bardi *et al.* 1958). In the present communication we discuss the identity of the next stable intermediate after porphobilinogen. Our evidence suggests that uroporphyrinogen is an intermediate, and that porphobilinogen does not form a corrole ring directly. Previous evidence for the contrary may have resulted from the choice of experimental material by past workers.

When choosing a micro-organism for the investigation of the biosynthesis of something that is formed in minute amounts, as with vitamin B_{12}, one naturally favours organisms that have an extraordinary ability to synthesize the desired compound. Thus propionibacteria, nocardia and actinomycetes have gained popularity as experimental material. However, these micro-organisms have a drawback for studies involving the question of the nature of the first tetrapyrrolic vitamin B_{12} intermediate, namely they all have the capacity to make haem enzymes. The question is raised whether a porphyrin formed by such organisms is a primordial haem molecule, or a primordial cobamide coenzyme.

Structural considerations make it apparent that no porphyrin occurring in the biosynthetic sequence after uroporphyrinogen can be considered as possible intermediates of vitamin B_{12}. The previous observation that vitamin B_{12}-producing organisms also produce coproporphyrin III tells us little beyond the fact that the organism has the capacity

to accumulate haem precursors under the conditions employed. There is no way of knowing if there is a branch at uroporphyrinogen.

At the outset of the present work it was therefore decided to avoid the ambiguities that arise with organisms that have multiple tetrapyrrole end products. The bacteria in the genus *Clostridium* seem to offer nearly ideal biosynthetic capabilities. They are known to synthesize reasonable quantities of vitamin B_{12} derivatives, and to date they have not been found to contain haem enzymes (Lascelles, 1964). They have never been observed to accumulate porphyrins. Since there is only one end product for δ-aminolaevulate in the clostridia it is assumed that any tetrapyrrole formed by the organisms from δ-aminolaevulate is a good candidate for consideration as a vitamin B_{12} intermediate.

Experimental. A culture of *Clostridium tetanomorphum* strain H1 was supplied by Dr H. A. Barker, and *Clostridium perfringens* by Dr. H. W. Seeley. They were grown in the media described by Barker *et al.* (1960), and by Meister, Sober & Tice (1951), respectively.

The work of Shemin, Corcoran, Rosenblum & Miller (1956) and that of Schwartz, Ikeda, Miller & Watson (1959) demonstrating that δ-aminolaevulate and porphobilinogen are active intermediates of vitamin B_{12} were carried out with actinomycetes. It seemed desirable at the outset therefore to determine whether the clostridia followed the pattern demonstrated in the earlier work. The results obtained with δ-amino[^{14}C]laevulate agree with the previous work. An experiment was also carried out to test the premise that clostridia do not make haem. This was done with ^{59}Fe. No detectable haem was found. Thus the preliminary evidence showed that clostridia use δ-aminolaevulate to make vitamin B_{12} and that vitamin B_{12} and its derivatives are the sole end product of δ-aminolaevulate metabolism in this organism.

Table 1. *Porphyrin formation by clostridia*

The incubation system contained 15 mg. of *C. tetanomorphum* or 5 mg. of *C. perfringens*, 125 μmoles of potassium phosphate buffer, pH 7·5, 5 μmoles of $MgCl_2$, 12·5 μmoles of fumarate, δ-aminolaevulate as indicated, cells and water to give a final volume of 2·5 ml. Incubation was at 37° under N_2 for 48 hr.

δ-Aminolaevulate added (μmole/ml.)	Porphyrin formed (μg./ml.)	
	With *C. tetanomorphum*	With *C. perfringens*
0	0	0
0·05	3·1	1·5
0·1	5·7	1·9
0·2	10·4	2·0
0·4	13·6	2·5
0·8	13·3	2·1

Experiments were then undertaken (see Table 1) to see if this organism could form and accumulate porphyrins under conditions where it could not make the complete B_{12} molecule. Accordingly *C. tetanomorphum* cell suspensions were incubated in two types of media. These were basically the same as Lascelles (1956, 1964) has used in her studies on porphyrin synthesis by the Athiorhodaceae. Mixture I contained glycine and succinate, and mixture II contained δ-aminolaevulate and fumarate. The incubations were carried out in Thunberg tubes at 37° under N_2. After I_2 oxidation, it was apparent that relatively large amounts of tetrapyrrole had been formed during the incubation with δ-aminolaevulate (Burnham, 1965). In addition, it was possible to see other pigmented compounds. Cells incubated with glycine and succinate did not make porphyrins.

The porphyrin was extracted from the suspension and was purified. It was identified as follows. On chromatography with Eriksen's (1958) solvent, the unknown porphyrin moved with the same R_F as uroporphyrin. The unknown porphyrin was esterified and chromatographed in the solvent system of Chu & Chu (1957) and it chromatographed with R_F identical with that of uroporphyrin octamethyl ester. The absorption spectrum of the ester ($\lambda_{max.}$ 502, 535, 572 and 626 mμ) was compared with those of authentic samples of other porphyrin esters and it showed absorption characteristics identical with uroporphyrin. On the basis of this evidence it is believed that the tetrapyrrole produced by *C. tetanomorphum* when incubated in the presence of δ-aminolaevulate is uroporphyrin. Similar experiments have been carried out with *C. perfringens* cell suspensions, and also with broken cell preparations of both micro-organisms, and in all cases uroporphyrin is the only porphyrin observed.

The other pigmented compound(s) produced in addition to uroporphyrin is currently under investigation. The formation of it tends to be erratic, whereas uroporphyrin formation is very regular. Its absorption spectrum resembles that of partially oxidized porphyrinogen, i.e. tetrahydroporphyrin, or dipyrrylmethene. However, the observed chemical properties are not consistent with those described by Mauzerall & Granick (1958) for such compounds.

Discussion. None of the evidence obtained in the present work indicates the point at which cobalt is incorporated. Suggestions have been made that ring-closure may occur around the cobalt atom (Bonnett *et al.* 1955). Porra & Ross (1965), however, have demonstrated that cell-free extracts of *C. tetanomorphum* catalyse the incorporation of cobalt into porphyrins, indicating that an enzyme exists which will catalyse the incorporation of cobalt into

macrocyclic tetrapyrroles and strongly suggesting that in the natural system the cobalt is incorporated into preformed tetrapyrrole.

The present evidence indicates that uroporphyrinogen is the first macrocyclic tetrapyrrole intermediate of vitamin B_{12}. It is suggested that the next step in the formation of the corrin ring is the reopening of the macro-ring with the expulsion of the γ-methene bridge carbon as methyl group, followed by ring-closure. Mauzerall (1960) has demonstrated that ring-opening does occur with uroporphyrinogens. The new compound, a methyl-substituted corrole, would not be expected to possess physical or chemical properties described for an unsubstituted corrole (Johnson & Kay, 1965). The substitution at the α-position of the A ring prevents the formation of a fully conjugated system as in corrole. Thus the characteristic Soret band and the typical red fluorescence would not be observed. A compound of the type suggested would, however, most likely resemble a tetrahydroporphyrin, or a di- or tri-pyrrylmethene. Since material with these absorption characteristics is sometimes found when clostridia are incubated with δ-aminolaevulate it is suggested that this material may represent the next biosynthetic intermediate of vitamin B_{12}.

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An Early Intermediate in the Biosynthesis of Biotin: Incorporation Studies with [^{35}S]Sulphate

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In a previous study on biotin biosynthesis, an unknown biotin vitamer, which was characterized as an amino acid, was shown to be present in the culture medium of *Phycomyces blakesleeanus* (Eisenberg, 1963). A compound with a similar R_F was also reported in culture filtrates of *Escherichia coli* (Dhyse & Hertz, 1958) and *Corynebacterium xerosis* (Genghof, 1956). This compound has now been obtained in high yields from the culture medium of a biotin auxotroph of *Penicillium chrysogenum*, and it becomes labelled when the organism is grown in the presence of [1,7-$^{14}C_2$] pimelic acid (M. A. Eisenberg, unpublished work). Since pimelic acid is a direct precursor of biotin (Eisenberg, 1962; Elsford & Wright, 1962), the isotope-incorporation studies suggested the possibility of this vitamer being an intermediate in biotin synthesis. To obtain more information about the chemical composition of this unknown vitamer, the question whether sulphur is present in the

compound became of paramount importance in view of the mechanism for biotin biosynthesis proposed by Lezius, Ringelmann & Lynen (1963). The present study with [^{35}S]sulphate shows no sulphur present in this unknown vitamer.

P. chrysogenum 62078 (strain obtained from the culture collection of Dr E. Tatum) was grown in a modified Czapek–Dox medium. The sulphate concentration was decreased to the lowest permissible level for maximum incorporation of [^{35}S]sulphate by substituting $MgCl_2$ for $MgSO_4$. To 500ml. of the media in a Fernbach flask was added 50mμg. of D-biotin and 100μc of $Na_2{}^{35}SO_4$. The $Na_2{}^{35}SO_4$ was purchased from New England Nuclear Corp. and had a specific activity of 1·33mc/mg. The maximum dilution of the specific activity by the non-labelled sulphate of the medium was 30-fold. A washed spore suspension from a 2-week-old culture grown on Sabouraud's medium was used to inoculate the medium. The flask was vigorously shaken on a New Brunswick incubator–shaker at 26° for 7 days. The mycelial mat was then removed by filtration and the filtrate adjusted to pH 2·2. Portions (5·0ml.) of the filtrate were chromatographed on the 0·9cm. × 15cm. or 0·9cm. × 50cm. columns of the Spinco model 120 amino acid analyser. The shorter column was developed with 0·35 N-sodium citrate buffer, pH 5·28, at 50° and the longer column with 0·38 N-sodium citrate buffer, pH 4·26, at 30° and 50°. The columns were connected directly to a Vanguard fraction collector and 5·0ml. samples taken. Each sample was assayed for biological activity by a modified disk-assay procedure of Genghof, Partride & Carpenter (1948) and for radioactivity with a Packard Tri-Carb scintillation counter.

Previous studies (M. A. Eisenberg, unpublished work) with the unknown vitamer on the columns of the amino acid analyser showed the unknown vitamer to appear between lysine and histidine on the short column at pH 5·28. On the medium column at pH 4·26, it appeared just beyond arginine. Fig. 1 shows that at pH 5·28 two biologically active peaks are obtained; the first consists primarily of dethiobiotin and the second of the unknown vitamer. Most of the radioactivity is eluted early with the neutral compounds and there is no coincidence between radioactivity and biological activity of the unknown vitamer. To further ascertain whether the small amount of radioactivity present was associated with the biologically active component, the contents of tubes 11–16 were pooled, the pH was adjusted to 2·2 and the solution was rechromatographed on the same column. Over 91% of the total biological activity and only 42% of the radioactivity was recovered, but again there was no indication of coincidence. The contents of those tubes containing biologically active material were

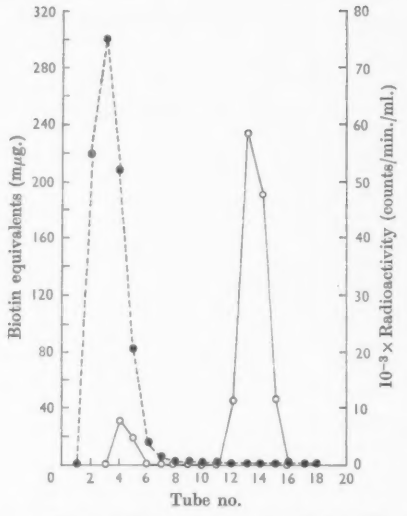

Fig. 1. Chromatographic separation of the unknown vitamer present in culture filtrates of *P. chrysogenum*. The column was developed with 0·35 N-sodium citrate buffer, pH 5·28, at 50°. ●—●, Radioactivity; ○—○, biological activity.

again pooled, the pH was adjusted to 2·2 and the solution was chromatographed on the medium column at pH 4·26. Under these conditions, over 80% of the biological activity was recovered and all the radioactivity lost. When the sample was placed directly on the medium column, only a few counts above background were found for each tube containing biologically active material and these values were not statistically significant.

It was previously postulated that pimelic acid may condense with a C_3 unit as the first step in the biosynthesis of biotin (Eisenberg, 1963). Of necessity, then, all subsequent intermediates would be labelled when cultures are grown in a medium containing ^{14}C-labelled pimelic acid. Evidence is now available that this unknown vitamer becomes labelled in the presence of [1,7-$^{14}C_2$]pimelic acid. In addition, when yeast cells are grown on the ^{14}C-labelled vitamer, the isotope is incorporated into biotin (M. A. Eisenberg, unpublished work). This suggests that the unknown vitamer is directly on the pathway of biotin biosynthesis. The absence of sulphur eliminates cysteine as the C_3 donor as postulated by Lezius *et al.* (1963), and indicates the incorporation of sulphur at a later stage as originally postulated by Tatum (1945). Further evidence in support of this late stage of sulphur incorporation is the finding of Pai

& Lichstein (1965) of the repression of biotin biosynthesis from dethiobiotin in resting cells of *E. coli* grown on biotin. This suggests that this reaction is directly on the biosynthetic pathway. A similar repression by biotin has been observed for the unknown vitamer and dethiobiotin in cultures of *P. chrysogenum*.

Two other C_3 donors that could possibly condense with pimelic acid are serine and alanine. The former could have readily arisen from cysteine via the cysteine-dehydratase reaction in the experiments of Lezius *et al.* (1963) and would have had a particularly high specific activity if serine synthesis via other pathways were repressed. Unfortunately, the specific activity of the serine in the proteins of the organism used was not checked. On the basis of the information presently available, the following scheme for biotin synthesis is proposed:

Serine or alanine + pimelic acid →→ unknown vitamer →→ dethiobiotin →→ biotin

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b

Short Communications

A Re-examination of the Molecular Structure of Yeast Glucan

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Although the presence of chains of β-(1→3)-linked D-glucose residues in yeast glucan is well established (Northcote, 1963), the nature and location of other glucosidic linkages is uncertain. On the basis of methylation analysis, Bell & Northcote (1950) suggested that the glucan was highly branched, had β-(1→2)-interchain linkages, and an average chain length of about 9 glucose residues. However, since on partial acid hydrolysis yeast glucan gave rise to a mixture of oligosaccharides containing both β-(1→3)- and β-(1→6)-linkages, Peat, Whelan & Edwards (1958*a*) concluded that the glucan was a linear molecule containing certain sequences of β-(1→3)- and β-(1→6)-linked glucose residues. The presence of (1→6)-linkages in yeast glucan was also confirmed by Peat, Turvey & Evans (1958*b*). We have therefore re-examined the minor structural features of yeast glucan, using a specimen (sample A) prepared by Dr W. D. Annan from the cell walls of baker's yeast according to the method of Bell & Northcote (1950). Our studies were facilitated by Dr D. J. Bell and Professor S. Peat, who kindly supplied samples of their respective preparations (samples B and P).

A branched glucan containing both β-(1→3)- and β-(1→2)-linkages should, on partial hydrolysis, yield laminaribiose and sophorose. The three samples of glucan (about 90mg.) were heated at 100° with 90% (w/w) formic acid for 30min., followed by 0·44N-H_2SO_4 for 1hr. The neutralized hydrolysates were fractionated by preparative paper chromatography, and the individual sugars methylated (by a micro-scale modification of the method of Kuhn, Trischmann & Low, 1955), methanolysed and examined by gas–liquid chromatography (Aspinall, 1963). Each oligosaccharide gave rise to the methyl glycosides of 2,3,4,6-tetra-*O*-methyl-D-glucose and of a tri-*O*-methyl-D-glucose, the identity of which enabled the oligosaccharide to be characterized. Authentic samples of laminaribiose, laminaritriose, gentiobiose and sophorose were similarly analysed.

All three samples of glucan gave similar products, namely glucose, laminaribiose, laminaritriose and gentiobiose. Sophorose was not detected, indicating that (1→2)-linkages were absent from the glucans, including sample B. The gentiobiose was not an artifact arising from acid-reversion, since pachyman, an insoluble linear β-(1→3)-glucan kindly provided by Professor W. J. Whelan (Warsi & Whelan, 1957), on partial acid hydrolysis under the above conditions, did not yield gentiobiose.

Glucan A (2g.) was methylated 16 times (with methyl sulphate and NaOH for the first six methylations, and methyl iodide and Ag_2O subsequently) to give a product, having 41·5% of OMe, in a final yield of 23%. Acid hydrolysis of a portion (250mg.) and fractionation on a cellulose column gave the following sugars, which were identified by gas–liquid chromatography of the methyl glycosides, and estimated by hypoiodite oxidation: 2,3,4,6-tetra-*O*-methyl-D-glucose, 6·4%; 2,4,6-tri-*O*-methyl-D-glucose, 63·4%; 2,3,4,-tri-*O*-methyl-D-glucose, 7·5%; 2,4-di-*O*-methyl-D-glucose, 8·3%; 4,6-di-*O*-methyl-D-glucose, 5·8%. In addition, the following sugars were tentatively identified: 3,4,6-tri-*O*-methyl-D-glucose, 0·8%; 2,6-di-*O*-methyl-D-glucose, 5·7%; mono-*O*-methyl-D-glucose and D-glucose, 2·1%. Although methylation was incomplete, the results clearly show that the molecule is branched, with a chain length of about 15 glucose units, and that about 7% of (1→6)-linked residues are present. Owing to undermethylation, the nature of the interchain linkages could not be unambiguously established, but the presence of 8·3% of 2,4-di-*O*-methyl-D-glucose indicates that a significant proportion of glucose residues are triply-linked at C-1, C-3 and C-6. The 3,4,6-tri-, 4,6-di- and lower methylated sugars are believed to arise from undermethylation and not to be structurally significant. The present methylation studies therefore confirm the results of Bell & Northcote (1950) with respect to the branched nature of the molecule, and those of Peat *et al.* (1958*a*) with regard to the presence of (1→6)-linked D-glucose residues.

The proportion of triol groups in glucan A was estimated from the amount of formic acid released on periodate oxidation. The results corresponded to the production of 1mol. of formic acid from

every 7 glucose residues; for glucans B and P, 1 mol. of formic acid was liberated from 10 glucose residues (Bell & Northcote, 1950; Peat *et al.* 1958*a*). The present result of 14% of triol groups is in good agreement with the combined yield of non-reducing end groups (6·4%) and (1→6)-linked glucose residues (7·5%) by methylation analysis.

Additional information on the arrangement of the two types of linkage was obtained by enzymic degradation studies. Incubation of glucan A with a bacterial laminarinase preparation kindly supplied by Glaxo Research Ltd. (Greenford, Middlesex) gave glucose, laminaribiose and laminaritriose, together with about 10% of an enzymically resistant and water-soluble glucan (limit dextrin); gentiobiose was not released. The enzyme preparation did not hydrolyse gentiobiose or the linear β-(1→6)-glucan, pustulan (see Lindberg & McPherson, 1954). The limit dextrin consisted largely of β-(1→6)-linked glucose residues since: (*a*) a partial acid hydrolysate was similar to that from pustulan, and contained a mixture of sugars with the R_{Glc} values of glucose, gentiobiose, gentiotriose and gentiotetraose; (*b*) by methylation analysis, the major tri-*O*-methyl-D-glucose formed was the 2,3,4-isomer; (*c*) the limit dextrin was slowly hydrolysed by a β-(1→6)-glucanase preparation from *Penicillium brefeldianum* QM1872, kindly supplied by Dr E. T. Reese and Dr M. Mandels.

It is therefore concluded that yeast glucan contains main chains of β-(1→6)-linked D-glucose residues to which are attached linear side chains of β-(1→3)-linked D-glucose residues. It seems probable that different samples of glucan differ in the degree of substitution of the main chain, and in the lengths of the side chains. For example, in a sample of glucan examined by Misaki & Smith (1963), almost all the β-(1→6)-linked D-glucose residues carry side chains that contain, on the average, 8 glucose residues. In sample A about half of the glucose residues in the main chains carry side chains, the average length of which is 15 glucose residues, whereas in sample B the side chains contain about 8 glucose residues (cf. Bell & Northcote, 1950).

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Studies on Methylazoxymethanol, the Aglycone of Cycasin: Methylation of Nucleic Acids *in vitro*

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Cycasin, the azoxyglucoside found in *Cycas circinalis* and *C. revoluta* (Nishida, Kobayashi & Nagahama, 1955; Riggs, 1956), is carcinogenic when fed to rats (Laqueur, Mickelsen, Whiting & Kurland, 1963). The active component is the aglycone, MAM* [$CH_3 \cdot N(O) : N \cdot CH_2 \cdot OH$], and not cycasin itself (Kobayashi & Matsumoto, 1965).

* Abbreviation: MAM, methylazoxymethanol.

There is a striking similarity in the lesions of rats given cycasin and those given dimethylnitrosamine (Laqueur, 1964). Dimethylnitrosamine affects primarily the endoplasmic reticulum of the liver cell (Emmelot & Benedetti, 1960). Liver cells of rats given cycasin show histological evidence of breakdown in the endoplasmic reticular structure, with accompanying decrease in liver RNA content (Williams, 1964). Liver nucleic acids are methyl-

ated *in vivo* by [^{14}C]dimethylnitrosamine (Magee & Farber, 1962).

Dimethylnitrosamine may be converted metabolically into the alkylating agent diazomethane (Magee & Hultin, 1962; Schoental, 1960), a carcinogen. MAM is isomeric with hydroxydimethylnitrosamine and thus it may also be transformed into diazomethane (Miller, 1964). Kriek & Emmelot (1963) used systems of aqueous solutions of microsomal or soluble RNA treated with diazomethane in ether, with vigorous stirring, to demonstrate methylation and breakdown of ribonucleic acids. The present communication reports on the methylation of nucleic acids and other compounds by MAM in a solution buffered at pH 7 at 37°.

MAM was prepared by the method of Kobayashi & Matsumoto (1965). RNA was obtained by the phenol extraction procedure of Kirby (1956) from the livers of six albino rats. The ultraviolet spectrum of the purified RNA showed a single peak at 258mμ. DNA was a commercial product obtained from calf thymus.

RNA (10mg.) was placed in a small test tube (12mm. × 125mm.) and mixed with 1m-mole (90mg.) of MAM in 1ml. of 0·2M-phosphate buffer, pH 7·0, and the mixture was kept at 37° for 16hr. RNA was then precipitated with 2 vol. of ethanol. The precipitate was sedimented in a centrifuge at 2000 rev./min. (480*g*), washed twice with ethanol–water (3:1, v/v) and dried in a vacuum desiccator over P_2O_5. The dried material was hydrolysed with 0·25ml. of N-HCl at 100° for 4hr. in a sealed tube (10mm. × 105mm.). An equal quantity of RNA was incubated without MAM and hydrolysed in the same manner.

DNA was treated in a similar manner with some modifications. The MAM-treated mixture was evaporated to dryness on a steam bath with the aid of a stream of air. The residue was hydrolysed with 0·5ml. of formic acid by the method of Wyatt (1950) at 175° for 1·5hr.

Samples (0·25ml.) of the hydrolysate were spotted on Whatman no. 1 paper (22cm. × 48cm.) and chromatographed by the descending method either in one or two dimensions. Three solvent mixtures, 2N-HCl in aq. 65% (v/v) propan-2-ol (Wyatt, 1951), butan-1-ol–0·6N-NH_3 (6:1, v/v) (Chargaff, Lipshitz, Green & Hodes, 1951) and butan-1-ol–formic acid–water (77:11:12, by vol.) (Markham & Smith, 1949), were used for the one-dimensional system and the last two solvent mixtures were used for the two-dimensional system (Weissmann, Bromberg & Gutman, 1957). The chromatograms were dried and the location of the bases was detected with ultraviolet radiation and outlined with pencil. The spots were cut out and the compounds were extracted by immersing the pieces of filter paper in 3ml. of 0·1N-HCl or 0·1N-NaOH for 20min. Blanks of equal sizes were cut out from adjacent spots where no compound was present. Ultraviolet spectra of the eluates were obtained with a Beckman DK-2 recording spectrophotometer.

A single methylated derivative, identified as 7-methylguanine, was found in the hydrolysates of both RNA and DNA treated with MAM. The derivative and some of the nucleic acid components were identified by comparing R_F values, and ultraviolet-absorption maxima reported by Reiner & Zamenhof (1957) and Brookes & Lawley (1961). Ultraviolet-absorption maxima and R_F values obtained for the derivative, authentic 7-methylguanine and guanine are shown in Table 1.

The ease with which MAM methylated nucleic acids indicated that it may be able to methylate other classes of compounds. The methylating action of MAM on phenol and *p*-chlorobenzoic acid was tested. Phenol (1m-mole) was dissolved in 2ml. of water, neutralized with 0·1N-NaOH and buffered with 2ml. of 0·2M-phosphate buffer. MAM (1m-mole) was added and the mixture was kept overnight at 37°, then extracted with 10ml. of diethyl ether. The ether was evaporated under reduced pressure and the residue was dissolved in 1ml. of chloroform. A portion of the chloroform solution (1μl.) was injected into an Aerograph Hi-Fi 600-D gas–liquid chromatograph with a hydrogen flame detector. The column was packed with 15% diethylene glycol succinate on Chromosorb W treated with hexamethyldisilazane. The flow of H_2 and the carrier gas, N_2, was maintained at 25ml./min. The column temperature was 103° and the injector temperature was 153°. A chromatogram with a single peak was obtained. Authentic anisole (1m-mole) was dissolved in 1ml. of chloroform and chromatographed in the same manner. The retention times of anisole and the derivative prepared by the treatment of phenol with MAM were identical. The yield of anisole produced with MAM was 5%. Riggs (1965) has demonstrated that, with a more vigorous reaction, anisole is produced in 40–50% yield when a solution of cycasin in molten phenol is treated with a drop of conc. H_2SO_4 and warmed until evolution of gas ceased.

p-Chlorobenzoic acid (1m-mole) was dissolved in 1ml. of 1·0N-NaOH and buffered with 0·2M-phosphate buffer. MAM (1m-mole) was added to the mixture, which was then kept at 37° for 16hr. A strong odour of an ester was detected at the end of the incubation period. The mixture was extracted once with 5ml. of chloroform. The chloroform was dried over Na_2SO_4 and the volume was reduced by evaporating the solvent under reduced pressure. The chloroform extract was chromatographed in the same manner as for anisole and a chromatogram with a single peak was obtained.

Table 1. *Ultraviolet-absorption maxima and R_F values of derivative from MAM-treated nucleic acids, 7-methylguanine and guanine*

Solvent 1, butan-1-ol–NH_3; solvent 2, butan-1-ol–formic acid; solvent 3, propan-2-ol–HCl.

	$\lambda_{max.}$ (mμ)		R_F		
Substance	In 0·1 N-HCl	In 0·1 N-NaOH	In solvent 1	In solvent 2	In solvent 3
Derivative from RNA	249	280	0·19	0·21	0·37
7-Methylguanine	249	280	0·18	0·20	0·38
Guanine	248	274	0·13	0·14	0·26
Derivative from DNA	249	280	0·19	0·21	
7-Methylguanine	249	280	0·19	0·21	
Guanine	248	273	0·15	0·16	

The chromatogram was compared with methyl *p*-chlorobenzoate prepared by the methanol–HCl esterification procedure of Stoffel, Chu & Ahrens (1959). The retention times of the products obtained by the esterification of *p*-chlorobenzoic acid with MAM and with methanol were identical. The yield of the ester prepared with MAM was 30%.

MAM is a good alkylating agent in spite of its instability in aqueous solution (Kobayashi & Matsumoto, 1965). The formation of 7-methylguanine is in accord with the action of diazomethane (Kriek & Emmelot, 1963) and dimethyl sulphate (Reiner & Zamenhof, 1957) on nucleic acids. The 7-position of guanine is the most readily alkylated in nucleic acids (Brookes & Lawley, 1961). The mild conditions of pH and temperature under which MAM is able to methylate suggests that diazomethane could be an intermediate in the reaction. The ease with which MAM methylates phenate and carboxylate groups indicates that methylated derivatives of cellular constituents other than purine bases can be expected in tissues treated with MAM *in vivo*.

Teas, Sax & Sax (1965) have demonstrated the radiomimetic effect of cycasin as measured by the frequency of induction of chromosome aberrations in root-tip cells of *Allium cepa*. Presumably, cycasin was hydrolysed by β-glucosidase in the plant and the active constituent was MAM. D. W. E. Smith (personal communication) has found that several histidine-requiring *Salmonella typhimurium* strains reverted to the wild type when treated with MAM–acetate. Thus it is probable that chemical action of MAM *in vivo* results in methylation of nucleic acid bases and induces chromosome aberrations, mutagenesis and carcinogenesis.

Alkylating agents in general are not considered powerful carcinogens (Brookes & Lawley, 1964), with the probable exception of dialkylnitrosamines and MAM. The use of dialkylnitrosamines in the study of carcinogenesis is limited to experiments *in vivo* as it requires enzymic conversion into the active principle. MAM can be used in isolated systems under mild conditions and shows promise as a compound useful in the study of carcinogenesis.

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Hypercholesterolaemia and Liver Enlargement in Experimental Hepatic Porphyria

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Schwartz (1955) was the first to describe a disturbance of lipid metabolism in experimental porphyria: he noted a significant increase of liver lipid phosphorus and total lipids in rats treated with Sedormid (2-isopropylpent-4-enoylurea). Labbe, Hanawa & Lottsfeldt (1961) later reported that the synthesis of fatty acids by the liver of rats injected with the related drug 2-allyl-2-isopropylacetamide was increased more than twofold and postulated the existence of a relationship between haem and lipid metabolism in the liver of porphyric animals. Taddeini, Nordstrom & Watson (1964) have found that the administration of 2-allyl-2-isopropylacetamide and also of 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine, another drug that induces porphyria, results in a marked elevation of serum cholesterol, total lipids and phospholipids in the rabbit. A considerable increase in liver size was also noted in their animals, a finding already reported by several other authors in experimental hepatic porphyrias caused by various drugs. The finding of hypercholesterolaemia together with increased porphyrin formation in rabbits made porphyric by two unrelated drugs and also in patients with hepatic porphyria (Taddeini *et al.* 1964) suggested the existence of a close relationship between porphyrin and lipid metabolism in porphyria.

Granick (1963) has shown that the drugs that cause experimental porphyria induce the formation of increased amounts of the liver enzyme δ-aminolaevulate synthetase, but the exact mechanism involved in this biochemical effect and the primary site of action of these drugs are not known. The association of other metabolic changes with porphyria may indicate that the synthesis of several liver enzymes is stimulated by these drugs and that porphyria is only one aspect of a more basic alteration involving liver protein biosynthesis (De Matteis & Gray, 1965). The main purpose of the present work was to establish whether porphyria, hypercholesterolaemia and liver enlargement, as well as the increased activity of certain liver enzymes (see below), are closely related phenomena and can be considered as effects resulting from one single action of these drugs.

Griseofulvin (7-chloro-2′,4,6-trimethoxy-6′-methylgris-2′-ene-3,4′-dione) causes a very marked hepatic and erythropoietic porphyria in mice (De Matteis & Rimington, 1963). In this work two compounds structurally related to griseofulvin, the isomer 7-chloro-4,4′,6-trimethoxy-6′-methylgris-3′-ene-2′,3-dione (isogriseofulvin) and the 2′-β-hydroxyethyl thioether analogue of griseofulvin (HET-griseofulvin) have been tested for the production of porphyria and other metabolic changes in male albino mice and rats.

In the mouse experiments the drugs were finely powdered and incorporated into diet M.R.C. 41B at a concentration of 1%. Animals were treated for 5–8 days. Faecal protoporphyrin was estimated during the treatment and liver protoporphyrin after the animals had been killed. Heparin-treated plasma from pooled blood of several animals was analysed for total cholesterol by either the method of Zlatkis, Zak & Boyle (1953) or that of Abell, Levy, Brodie & Kendall (1952), and also for lipid phosphorus concentration (Fiske & Subbarow, 1925). After 8 days of treatment mice were given access to normal diet for 24hr. and then injected intraperitoneally with 150mg. of hexobarbitone sodium/kg. body wt.: their 'sleeping time' was compared with that of control mice to assess the activity of the drug-metabolizing microsomal enzymes in the oxidation of the barbiturate *in vivo*.

It was found (Table 1) that isogriseofulvin was much more active than griseofulvin in inducing porphyria, whereas HET-griseofulvin was practically inactive. However, liver enlargement, elevation of plasma cholesterol and lipid phosphorus concentration and decrease of the hexobarbitone 'sleeping time' were observed after treatment with all three drugs. A marked decrease of the hexobarbitone 'sleeping time' was also observed in mice injected subcutaneously with 2-allyl-2-isopropylacetamide (200mg./kg. body wt./day) for 3 days.

In starved male albino rats, a considerable increase in liver size and a significant hypercholesterolaemia were observed after the oral administration of griseofulvin or either of its two analogues (2g./kg. body wt./day) or after subcutaneous injection of 2-allyl-2-isopropylacetamide (300mg./kg. body wt./day) for 3 days. 2-Isopropyl-2-propylacetamide and chloretone (1,1,1-trichloro-2-methylpropan-2-ol) also produced liver enlargement and hypercholesterolaemia, but to a smaller extent. The

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Table 1. *Effect of the administration of griseofulvin, isogriseofulvin or HET-griseofulvin (1% in the diet) on liver size and protoporphyrin content, plasma cholesterol and lipid phosphorus concentrations and hexobarbitone 'sleeping time' of male albino mice*

The values are the means ±S.E.M. of the numbers of observations in parentheses. *$P<0.025$, **$P<0.01$, ***$P<0.001$, where P (obtained by Student's t test) is the probability that the observed differences from control values could have arisen by chance.

	Liver wt. (g./100g. body wt.)	Liver protoporphyrin (μg./g. wet wt. of liver)	Plasma cholesterol (mg./100ml.)	Plasma lipid phosphorus (mg./100ml.)	Hexobarbitone 'sleeping time' (min.)
Control	5·31±0·27 (10)	<0·6† (6)	125·0± 8·8 (9)	6·73±0·1 (9)	65·4±4·2 (12)
Griseofulvin	7·10±0·56 (6)*	23·6±11·6 (6)***	204·9±29·9 (4)*	11·77±2·3 (4)*	35·8±5·3 (6)***
Isogriseofulvin	9·36±0·66 (6)**	311·8±61·2 (5)***	188·4±15·8 (4)*	10·89±0·9 (4)**	26·0±4·5 (12)***
HET-griseofulvin	9·29±0·16 (9)***	<0·6† (6)	194·0±16·5 (10)**	9·49±0·9 (9)*	28·4±2·8 (13)***

† The value of 0·6μg./g. wet wt. of liver is the lowest concentration of protoporphyrin that can be determined with accuracy in the method employed.

last two drugs and HET-griseofulvin did not, however, cause any appreciable increase in urinary porphobilinogen excretion.

Some additional investigations were carried out in rats made porphyric by 2-allyl-2-isopropylacetamide treatment. A 30–50% increase in the activity of the liver glucose 6-phosphate dehydrogenase and UDP-glucose dehydrogenase (per mg. of protein) was observed after treatment with the drug. Treated animals also showed an approx. 50% increase in the size of the adrenals. Actinomycin D injected at the dose of 250μg./kg. body wt./day together with 2-allyl-2-isopropylacetamide produced a considerable inhibition of porphyria, but did not influence the degree of liver enlargement due to the drug and caused a further increase in the concentration of blood cholesterol. This is similar to the results obtained for the increased L-ascorbic acid excretion after 2-allyl-2-isopropylacetamide treatment, which can also be dissociated from porphyria by treatment with actinomycin D (De Matteis, 1964). It was also found that the administration of actinomycin D alone produced a considerable hypercholesterolaemia and a significant enlargement of the adrenals, but no increase in liver size.

These results show that a complex metabolic reaction follows the administration of several chemically unrelated drugs and that one single drug, such as 2-allyl-2-isopropylacetamide, can cause a rise in the activity of several different liver enzymes. The present findings also suggest that liver enlargement and elevation of plasma lipids are not closely related to the disturbance of porphyrin metabolism and may result from different actions of the drugs; both these changes are also produced by drugs that do not cause porphyria and their association with increased porphyrin formation should probably be regarded as coincidental. The same is likely to be true for the increased activity of drug-metabolizing enzymes and for the enhanced excretion of L-ascorbic acid (De Matteis, 1964) that also occur in experimental porphyria.

In contrast with these metabolic changes produced by many chemically unrelated drugs, porphyria appears to be a more specific effect caused only by some drugs, probably those possessing some particular structural configuration. Studies carried out with compounds chemically related to 2-allyl-2-isopropylacetamide (Goldberg & Rimington, 1955) and to 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (Marks, Hunter, Terner & Schneck, 1965) have shown the existence within both groups of a structural requirement for the induction of porphyria. The results of the present work show that this is also the case for the griseofulvin group of drugs.

The mechanism involved in the production of hypercholesterolaemia by these drugs is not known. Increased hepatic synthesis of cholesterol and other lipids may result from increased activity of liver enzymes concerned in their biosynthetic pathways.

I am grateful to Professor C. Rimington, F.R.S., for his interest in this work, to Dr D. G. Davey for a gift of griseofulvin and analogues and to Merck, Sharp and Dohme (Rahway, N.J., U.S.A.) for a gift of actinomycin D. The skilled technical assistance of Mr M. Ally is also acknowledged.

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Short Communications

Evidence for the Metabolism of Glycerol by Skeletal Muscle and the Presence of a Muscle Nicotinamide–Adenine Dinucleotide Phosphate-Dependent Glycerol Dehydrogenase

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The absence of a readily demonstrable glycerol kinase in muscle (Wieland & Suyter, 1957) has been generally regarded as indicating an inability of muscle to metabolize glycerol (Randle, Garland, Hales & Newsholme, 1963; Garland & Randle, 1964). There is, however, one report of the isolation of [^{14}C]glycogen from muscle that had been incubated in a medium containing [^{14}C]glycerol (Bloom & Foster, 1964).

In attempts to obtain further information on the ability of muscle to metabolize glycerol, [1,3-$^{14}C_2$]glycerol was incubated with rat diaphragm muscle and the radioactivities of the CO_2 produced during the incubation and of the lipids extracted from the tissue and medium after the incubation were measured.

In Table 1 the results of [^{14}C]glycerol metabolism in skeletal muscle are given. It is evident that some of the [^{14}C]glycerol has been converted into $^{14}CO_2$. If it is assumed that the respiratory quotient of muscle in the absence of added substrates (apart from glycerol) is 0·8 (Long, 1961), approx. 1·3% of the total CO_2 produced could have been derived from glycerol. The average incorporation of [^{14}C]-glycerol/hr./100mg. wet wt. of muscle into CO_2 is 0·113% and into lipids is 0·106%. In a few experiments liver slices were also incubated to determine the relative ability of the two tissues to metabolize glycerol to CO_2. In several experiments measurements were made of the concentration of glycerol in the incubation medium before and after the 60min. incubation. With the liver slices there was a slight fall in the glycerol concentration of the medium, whereas with diaphragm the glycerol concentration of the medium doubled after the 60min. of incubation. This indicates that glycerol was being released from the muscle during the incubation, presumably derived from triglyceride hydrolysis. Whereas much less $^{14}CO_2$ was formed from the glycerol in muscle than in liver (Table 1), the isotope dilution of glycerol in the medium containing muscle suggests that the rate of metabolism of glycerol to CO_2 in muscle is probably similar to the rate of its conversion into CO_2 in liver.

Though the results in Table 1 establish that glycerol is metabolized in muscle they provide no indication of any pathway for its metabolism. Several attempts to demonstrate the presence of a glycerol kinase in muscle were unsuccessful. However, during an investigation of all conceivable ways by which glycerol might enter the metabolic processes in muscle, the presence of an NADP-specific glycerol dehydrogenase was established. The enzyme was found predominantly in the non-particulate fraction of the cell. It is precipitated by between 50% and 70% saturation with $(NH_4)_2SO_4$. Partial purification was achieved by passing the resuspended 50–70% saturated $(NH_4)_2SO_4$ precipitate through Sephadex G-100. The R_F of muscle glycerol dehydrogenase on Sephadex G-100 was 2·04, suggesting a mol. wt. of approx. 15000 (Whitaker, 1963).

Because the equilibrium for muscle glycerol dehydrogenase was found to be in the direction of glycerol formation ($K_{eq.} = 1{\cdot}6 \times 10^{-3}$ at pH 7·0) most enzyme assays were made by measuring the oxidation of NADPH with glyceraldehyde as substrate. Kinetic measurements were made at 340mμ with a Unicam SP. 800 spectrophotometer equipped with a variable-speed slave recorder with a full-scale expansion of 0–0·1 extinction unit. The incubation was carried out at 25° in quartz cuvettes of 1cm. light-path. The incubation medium contained sodium phosphate buffer, pH 7·0 (50mM), in a total volume of 3·0ml. D-Glyceraldehyde, NADPH and glycerol dehydrogenase were present in various concentrations depending on the experiment. The control cuvette contained all reagents except D-glyceraldehyde. The reaction was started by the addition of enzyme to the cuvettes containing all the other components.

Because the double-reciprocal plots of initial velocity versus concentration of D- or DL-glyceraldehyde could be superimposed when the calculation for the DL-racemate was made for the D-isomer only, it was concluded that the enzyme was specific for D-glyceraldehyde. Dihydroxyacetone was reduced at 5% of the rate of D-glyceraldehyde. The rate with NADH was only 10% of that with an equivalent amount of NADPH. The pH optimum

Table 1. *Formation of* $^{14}CO_2$ *and* ^{14}C*-labelled lipids from* [^{14}C]*glycerol*

The diaphragm pieces from male hooded rats (Quebec Breeding Farms, St Eustache, Quebec, Canada) were incubated in Warburg flasks containing: 2·5ml. of a low-calcium (55mM) Krebs–Ringer phosphate buffer, pH7·4 (Krebs & Henseleit, 1932), glycerol (3·3mM), 1μc of [1,3-$^{14}C_2$]glycerol (0·16mc/mg.; New England Nuclear Corp., Boston, Mass., U.S.A.) and rat tissue slices (100–200mg.) in a total volume of 3·0ml. The glycerol substrate was in one side arm and 0·2ml. of 50% (w/v) citric acid in the other. The filter paper in the centre well was moistened with 0·1ml. of 33% (w/v) KOH. After 15min. of preincubation at 37° the glycerol was added from the side arm. The contents of the control flasks were identical with those of the experimental flasks, but the tissue was boiled before its addition to the flasks. Oxygen uptake was measured at 5min. intervals for 60min. Citric acid was then added from the side arm to liberate all the CO_2 from the buffer. The contents of the centre well were quantitatively transferred to the main compartment of another Warburg flask containing 0·2ml. of Hyamine hydroxide (1·0M-di-isobutylcresoxyethoxyethyldimethylbenzylammonium hydroxide in methanol; Rohm and Haas Co., Philadelphia, Pa., U.S.A.) in the centre well and 0·2ml. of 7·1N-H_2SO_4 in the side arm. After the system was closed the H_2SO_4 was added to the main compartment and the flasks were shaken for 40min. The Hyamine hydroxide was then transferred to a scintillation vial containing Liquifluor–toluene [Pilot Chemicals Inc., Watertown, Mass., U.S.A.; the Liquifluor was diluted in toluene to give a concentration of 4g. of 2,5-diphenyloxazole and 50mg. of 1,4-*p*-bis-(5-phenyloxazol-2-yl)benzene/l.] scintillation solution. Lipids were extracted with chloroform–methanol (Folch, Lees & Sloane-Stanley, 1957). After evaporation to dryness the lipids were dissolved directly in 10ml. of Liquifluor–toluene scintillation fluid. The samples were counted in a Nuclear–Chicago scintillation counter (model 724). The efficiency was determined by the channels-ratio method (Bush, 1963).

	Radioactivity in CO_2* ($\mu\mu$c)				Radioactivity in muscle lipids* ($\mu\mu$c)	
Tissue slices in experimental flasks	Experimental flask (per 100mg. of tissue)	Control flask	Percentage of [^{14}C]-glycerol converted into $^{14}CO_2$	Percentage of total CO_2 derived from glycerol	Experimental flask (per 100mg. of tissue)	Control flask
Diaphragm (200mg.)	945	45	0·09	0·98	—	—
Diaphragm (280mg.)	367	45	0·032	0·36	—	—
Diaphragm (110mg.)	1990	183	0·18	1·3	—	—
Diaphragm (120mg.)	1460	183	0·13	1·0	—	—
Diaphragm (170mg.)	1340	226	0·11	1·3	—	—
Diaphragm (100mg.)	520	264	0·03	0·4	—	—
Diaphragm (80mg.)	704	3	0·07	2·6	—	—
Diaphragm (160mg.)	1560	3	0·16	4·6	—	—
Diaphragm (190mg.)	340	212	0·0012	0·16	186 (0·017)‡	14
Diaphragm (230mg.)†	1480	212	0·012	0·67	1470 (0·14)‡	14
Diaphragm (100mg.)†	—	—	—	—	1670 (0·16)‡	58
Diaphragm (170mg.)	4320	37	0·43	—	—	—
Liver (220mg.)	2190	169	0·21	3·2	—	—
Liver (190mg.)	3440	167	0·32	3·0	—	—
Liver (190mg.)	8850	303	0·85	—	—	—

* Counts/min. were calculated to 100% efficiency by dividing by the efficiency obtained by channels-ratio counting.

† Glycerol 0·8mM.

‡ Percentage of [^{14}C]glycerol incorporated into lipids.

is 7·0 for D-glyceraldehyde reduction and 9·0 for glycerol oxidation. The K_m values for the reactants are: D-glyceraldehyde, $4{\cdot}5\times10^{-5}$M; NADPH, $1{\cdot}39\times10^{-5}$M; glycerol, 0·1M; NADP, $1{\cdot}25\times10^{-5}$M. The K_m values, however, are somewhat dependent on the concentration of the fixed substrate. No bivalent metal requirement for the enzyme could be demonstrated, and EDTA is not inhibitory at a concentration of 10mM. Glycerol dehydrogenases that are NADP-dependent have been described in micro-organisms (Chiang & Knight, 1959; Rao, Ramakrishnan & Sirsi, 1960) and also in rat liver (Moore, 1959).

The present findings indicate that glycerol can be metabolized in skeletal muscle. The presence of a glycerol dehydrogenase suggests that the initial step of glycerol metabolism may be the formation of D-glyceraldehyde. Fructose 1-phosphate might then be formed by the action of the muscle aldolase to condense D-glyceraldehyde with dihydroxyacetone phosphate. It could then be possible for fructose 1,6-diphosphate, a normal

glycolytic intermediate, to be formed by the action of a specific fructose 1-phosphate kinase (Slein, Cori & Cori, 1950). It is, however, difficult to speculate on the activity of the enzyme under conditions obtaining in intact muscle owing to the lack of knowledge of steady-state concentrations of glyceraldehyde and of glycerol in muscle cytoplasm. In phosphate buffer, pH 7·4 (0·05M), with saturating concentrations of D-glyceraldehyde and NADPH the glycerol dehydrogenase in homogenates of skeletal muscle metabolized 2800mμ-moles of D-glyceraldehyde/hr./g. of muscle. Under optimum conditions, without provision for the removal of the glyceraldehyde, the rate of the reaction in the reverse direction (glycerol to D-glyceraldehyde) is one-tenth of this. The rate of [^{14}C]glycerol conversion into $^{14}CO_2$ (113mμmoles/hr./g. of muscle) and incorporation into lipids (29mμmoles/hr./g. of muscle) in diaphragm slices (Table 1) is therefore apparently of a similar order of magnitude to the activity of glycerol dehydrogenase in skeletal-muscle homogenates.

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The Conversion of 7-Dehydrocholesterol into Cholesterol

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Previous investigations from several Laboratories have established the sequence of reactions leading to synthesis of cholesterol (II) from acetate (Clayton, 1965). The mechanisms of a number of the reactions, especially those between acetate and lanosterol, have also received attention (Clayton, 1965). However, details of the reactions that result in the formation of cholesterol from lanosterol are still obscure. In the biosynthesis of cholesterol from lanosterol three methyl groups at positions 14, 4α and 4β are replaced by three hydrogen atoms, the $\Delta^{24,25}$-double bond is reduced and the $\Delta^{8,9}$-double bond is replaced by one between $C_{(5)}$ and $C_{(6)}$.

Some recent reports suggest that the last intermediate in the biosynthesis of cholesterol (II) is 7-dehydrocholesterol (I) (Dempsey, Seaton, Schroepfer & Trockman, 1964; Dvornik, Kraml & Bagli, 1964); however, it has not yet been unambiguously established that 7-dehydrocholesterol is an obligatory intermediate in the biosynthesis of cholesterol.

The present communication describes the origin and stereochemistry of the two hydrogen atoms at positions 7 and 8 during the conversion of 7-dehydrocholesterol (I) into cholesterol (II) with rat-liver homogenates by the enzyme 7-dehydrocholesterol reductase. Optimum conditions for the conversion of 7-dehydrocholesterol (I) into cholesterol (II) were studied by using 7-dehydro[19-^{3}H]cholesterol (Akhtar & Gibbons, 1965). In all experiments

cholesterol was isolated by preparative thin-layer chromatography and purified to constant radioactivity by the dibromide method (Fieser, 1953).

Incubation of non-radioactive 7-dehydrocholesterol (I) in the presence of [4-^{3}H]NADPH (Popják, Goodman, Cornforth, Cornforth & Ryhage, 1961) gave radioactive cholesterol. Although there were individual variations, the direct transfer of hydrogen from NADPH to cholesterol was demonstrated by six independent experiments. The position of the hydrogen atom thus biosynthesized was established by the following sequence of reactions.

Cholesterol (II), on treatment with acetic anhydride, gave the acetate (III), which on treatment with $NaNO_2$ and HNO_3 yielded the nitro compound (IV). On reduction with zinc and acetic acid at 80° the nitro compound gave the ketone (V), which retained 88% of the radioactivity of the parent acetate (III). The ketone (V), when treated with methanolic KOH, lost at least 93% of the activity of the original acetate (III). These experiments conclusively establish that in the conversion of 7-dehydrocholesterol (I) into cholesterol (II) one hydrogen atom is transferred directly from NADPH to position 7 of cholesterol.

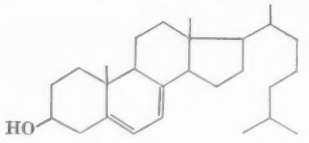
In the second series of experiments non-radioactive 7-dehydrocholesterol (I) was incubated in the presence of tritiated water and non-radioactive NADPH. The cholesterol was processed to the ketone (V) as described above. Radioactivity measurement of the base-equilibrated ketone (V) revealed that the cholesterol biosynthesized in the presence of tritiated water contained 74% of the radioactivity at $C_{(7)}$ and hence the remaining 26% at $C_{(8)}$. Since we have already shown that the hydrogen at $C_{(7)}$ is derived from NADPH, the presence of radioactivity at $C_{(7)}$ in the tritiated-water experiment remains to be explained. It has been suggested by Popják *et al.* (1961) that there is a soluble enzyme system in rat-liver homogenates that directs an equilibration of hydrogen at $C_{(4)}$ of NADPH with those of water by the following sequence of reactions:

$$NADPH + R + H^+ \rightleftharpoons NADP^+ + RH_2 \quad (1)$$

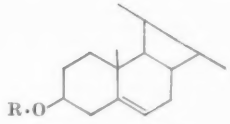
$$RH_2 + T_2O \rightleftharpoons RT_2 + H_2O \quad (2)$$

$$RT_2 + NADP^+ \rightleftharpoons R + NADPT + T^+ \quad (3)$$

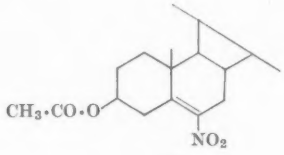
The radioactivity at $C_{(7)}$ in cholesterol (Expt. 2 in Table 1) biosynthesized in the presence of



(I)



(II) R=H
(III) R=CH$_3$·CO



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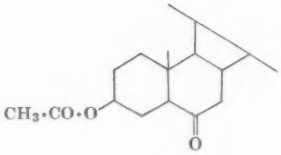
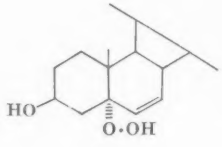
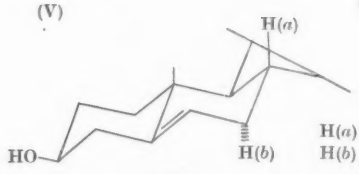


(V)



(VI)



H(a): from proton source
H(b): from NADPH

(VII)

Table 1. *Incorporation of* ^3H *into cholesterol from* [4-^3H]*NADPH and tritiated water on incubating 7-dehydrocholesterol with rat-liver homogenate*

Samples (10 ml.) of a 500*g* cell-free supernatant of rat-liver homogenates (1·25 ml. of 0·1 M-sodium phosphate buffer, pH 7·3/g. of liver) were incubated with 300 μg. of 7-dehydrocholesterol at 37° for 3 hr. under anaerobic conditions. In Expt. 1, 2 μmoles of [4-^3H]NADPH (6·5 μc) were used, giving cholesterol of activity 2500 counts/min. In Expt. 2, 2 μmoles of NADPH and 15 μmoles of glucose 6-phosphate together with 100 mc of tritiated water were used, giving cholesterol of activity 23 700 counts/min. The radioactive samples were dissolved in Nuclear Enterprise 213 or 220 liquid-scintillation fluid and counted in an I.D.L. 2022 liquid-scintillation counter at 15% efficiency. Experimental error was less than 3%.

	Activity of compounds (counts/min./m-mole)						
	Cholesterol (II)	6-Nitro compound (IV)	6-Oxo compound (V)	6-Oxo compound after base treatment (V)	5α-Hydroperoxy compound (VI)	Percentage of total radioactivity	
						At $C_{(7)}$	At $C_{(8)}$
Expt. 1:							
Cholesterol from [4-^3H]NADPH	5800	—	5120*	440	—	93	7†
Expt. 2:							
Cholesterol from tritiated water	9050	8800	7540*	2320	2520	74	26

* Loss due to partial equilibration under acid conditions.

† The corrected value for the radioactivity at $C_{(8)}$ in this experiment is considerably less than 7%. However, this correction involves a few assumptions and therefore we prefer to quote the uncorrected value.

tritiated water can only have originated from NADPH formed by the equilibration reaction (3). The equilibration reaction (3) does not affect the main conclusion based on the [4-^3H]NADPH experiment because any equilibration of the label in this experiment would hardly affect the net radioactivity of the hydrogen atoms of water. The equilibration reaction, however, decreases the incorporation of label from [4-^3H]NADPH into cholesterol (II).

We now attempted to determine the stereochemistry of the hydrogen at $C_{(7)}$. Cholesterol obtained from the tritiated-water experiment was converted into the 5α-hydroperoxide by the method of photo-oxidation (Nickon & Bagli, 1961). This conversion resulted in a 97% loss of label originally present at $C_{(7)}$ (deduced from Table 1). It has been shown (Nickon & Bagli, 1959) that in the conversion of cholesterol into the hydroperoxide (VI) the 7α-hydrogen is stereospecifically lost. We therefore conclude that in the conversion of 7-dehydrocholesterol (I) into cholesterol (II) the 7α- and 8β-hydrogen atoms are derived from NADPH and a suitable proton source respectively. The overall addition is *trans*-diaxial, as shown in formula (VII).

The relative isotope effect, 3:1, in the formation of $C_{(8)}$–H and $C_{(7)}$–H bonds respectively, suggests that the crucial step in the 7-dehydrocholesterol reductase-catalysed conversion of 7-dehydrocholesterol (I) into cholesterol (II) is the formation of the $C_{(8)}$–H bond, thus generating a highly stable allylic carbonium ion at $C_{(7)}$ that in accordance with chemical expectations is neutralized by the delivery of a hydrogen by NADPH from the α-side of the steroid molecule.

Avigan & Steinberg (1961) have shown that an NADP-dependent dehydrogenase is concerned in the reduction of the side-chain double bond between $C_{(24)}$ and $C_{(25)}$ of steroids, and our results suggest that in this reduction the hydrogen atoms at $C_{(24)}$ and $C_{(25)}$ would be derived from a proton source and NADPH respectively.

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Alteration in the Electrophoretic Mobility of Alkaline Phosphatases after Treatment with Neuraminidase

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Attempts to distinguish between the alkaline phosphatases (orthophosphoric monoester phosphohydrolases, EC 3.1.3.1) obtained from different human tissues have been based on variations in kinetic properties, reactions with certain inhibitors, and differences in immunological, chromatographic and electrophoretic behaviour. Of these criteria, differences in mobility on starch-gel electrophoresis have received considerable attention recently, and essentially comparable results of investigations of this type have been reported from several Laboratories (Wilkinson, 1965). The existence of electrophoretically distinct alkaline-phosphatase components in human blood serum has also been noted. In the absence of highly purified preparations of human alkaline phosphatase it has not been possible to decide whether these electrophoretic differences represent fundamental variations in molecular architecture between the enzymes from different sources, or whether they indicate differences that do not affect the primary structure of the enzyme molecule, e.g. due to interaction of the enzyme protein with non-enzymic entities.

A decrease in electrophoretic mobility after treatment of certain human blood serum proteins with neuraminidase (EC 3.2.1.18) has been demonstrated by Schultze (1962), and Robinson & Pierce (1964) showed that, of two alkaline-phosphatase components in human serum, one was retarded by neuraminidase treatment. The action of neuraminidase also converts the heterogeneous pattern seen on starch-gel electrophoresis of human-kidney alkaline phosphatase into a single active enzyme zone (Butterworth & Moss, 1966). The present report describes the effect of neuraminidase on the electrophoretic properties of human-liver and -intestinal alkaline phosphatase, both in unfractionated tissue extracts and after several purification stages.

Phosphatase-containing extracts of fresh post-mortem specimens of human liver and small intestine were made by Morton's (1950) butanol method. Further purification of the tissue extracts was achieved by gel filtration on a column of Sephadex G-150 (Pharmacia A.B., Uppsala, Sweden) followed by column chromatography on DEAE-cellulose with gradient elution at pH 7·7 (Grossberg, Harris & Schlamowitz, 1961). Tissue extracts or chromatographic fractions (0·1 ml.) were incubated with 0·4 ml. of neuraminidase solution (from *Clostridium perfringens*; Sigma Chemical Co. Ltd., type V: 1 mg./ml. in tris buffer, pH 7·7) for 24 hr. at 37°. Treated and untreated alkaline-phosphatase solutions were submitted to horizontal starch-gel electrophoresis (Smithies, 1955) in a discontinuous tris–citrate–borate buffer system at pH 8·6 (Poulik, 1957) for 17 hr. at an initial voltage of 6 v/cm. After electrophoresis, alkaline-phosphatase zones were located on the gel as described by Estborn (1959).

The electrophoretic mobility of the major alkaline-phosphatase zone of liver extract was considerably decreased by neuraminidase action, and a minor enzyme zone close to the origin was also retarded (Fig. 1*a*). Neuraminidase treatment had little effect on the electrophoretic pattern of intestinal alkaline phosphatase, only a small amount of activity being detected in the region corresponding to the main retarded zone of liver phosphatase (Fig. 1*b*). Similar effects were observed when the unfractionated tissue extracts were replaced by enzyme fractions partially purified by gel filtration and anion-exchange chromatography (Figs. 1*c* and 1*d*). The relative lack of effect of neuraminidase treatment on intestinal phosphatase was not due to incomplete action of neuraminidase as a result of inactivation; at the end of the 24 hr. incubation period the neuraminidase was still active, as was shown by adding liver extract and continuing the incubation, when the retarding effect of neuraminidase on liver phosphatase was still produced. This experiment also demonstrated that the lack of activity on intestinal extract was not due to the presence in the extract of factors that inhibited the neuraminidase action. In all experiments, the total alkaline-phosphatase activity was unaffected by neuraminidase treatment when compared with similarly incubated controls. Incubation with lysozyme and hyaluronidase, enzymes of related specificity, produced no effect on electrophoretic mobility.

The differential effect of neuraminidase on liver and intestinal alkaline phosphatases provides a further distinction between the latter phosphatase and those from other tissues. The effect does not

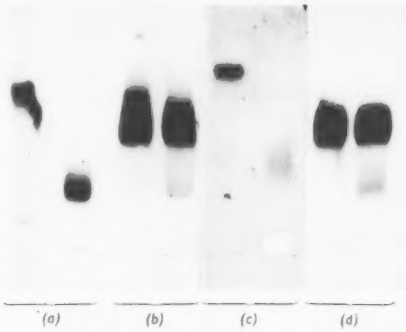

Fig. 1. Comparison of alkaline-phosphatase zones of human liver and small intestine on starch-gel electrophoresis at pH 8·6, showing the effect of incubation with neuraminidase. (*a*) Liver extract; (*b*) small-intestinal extract; (*c*) liver alkaline phosphatase after fractionation by gel filtration and anion-exchange chromatography; (*d*) intestinal alkaline phosphatase after fractionation by gel filtration and anion-exchange chromatography. In each case, the sample incubated with neuraminidase is on the right and the control incubated without neuraminidase on the left. The sample slots are near the bottom and the anode at the top.

seem to be due to impurities in the enzyme preparations, since it persists after several purification stages. These purification procedures remove low-molecular-weight substances and certain sialic acid-containing proteins such as orosomucoid and haptoglobin, which, by interacting with alkaline phosphatase, might give rise to the effect seen on treatment with neuraminidase. The present findings agree with those of Robinson & Pierce (1964) that a phosphatase zone of human serum, which was presumed to arise from intestine since it was inhibited by L-phenylalanine (Fishman, Green & Inglis, 1962), was not retarded on starch-gel electrophoresis after neuraminidase treatment, and with the observation of Engström (1961), who detected no sialic acid in purified calf-intestinal alkaline phosphatase. The small effect of neuraminidase on intestinal alkaline phosphatase may be due to the presence of a second, sialic acid-containing, phosphatase; it was, however, seen when chromatographic fractions of intestinal phosphatase were used (Fig. 1*d*), as well as with unfractioned tissue extracts, suggesting that fractionation had failed to remove any such second component.

The slow-moving phosphatase zone near the origin in liver extracts has been attributed to formation of a complex of the phosphatase with lipoprotein (Moss, 1962). The retardation of this zone by neuraminidase treatment supports this suggestion, since removal of sialic acid residues would be expected to affect the electrophoretic mobility of such complexes as well as that of the free enzyme molecules, provided that the sialic acid residues were not rendered inaccessible to neuraminidase action by the structure of the complex.

The present findings support the implication of other studies that intestinal alkaline phosphatase differs from phosphatases from other tissues, and they indicate a possible basis for the different electrophoretic mobilities of these enzymes.

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The Use of Acrylonitrile for the Subtractive Identification of the *N*-Terminal Residues of Small Peptides

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The elucidation of the primary structures of proteins by chemical methods depends on the determination of the amino acid sequences of peptides produced by more or less specific methods of hydrolysis. This is usually achieved by submitting these peptides to additional partial hydrolytic procedures and separating and characterizing the smaller peptides produced. The separations are generally carried out by paper electrophoresis or paper chromatography, and the peptides may only be available in submicromolar amounts. There are several methods that are sufficiently sensitive and accurate for the determination of the amino acid composition of such small quantities of a peptide, but no entirely satisfactory method is yet available for the determination of its *N*-terminal residue. The established methods of Sanger (1945) and Edman (1950) are too insensitive to be used at this level. Radiochemical modifications of these methods, and the 'pipsyl' method (Levy, 1957; Corfield, Dilworth, Fletcher & Gibson, 1959), make excessive demands on technique and equipment. The 'DNS' method (Gray & Hartley, 1963) is sensitive enough and extremely valuable, but it is not always reliable, possibly owing to the slowness of the labelling reaction for certain peptides in comparison with the rate of hydrolysis of the reagent. The use of the Edman degradation as a subtractive method on the submicromolar scale has been described by Bahl & Smith (1965), but there exists a need for a simple, sensitive and reliable method for use when stepwise degradation of a peptide is not necessary.

The reaction between acrylonitrile and peptides provides a basis for such a method: addition takes place readily in the presence of bases (Friedman & Wall, 1964), the excess of reagent is easily removed and the *N*-carboxyethyl derivatives that are produced by hydrolysis of the *N*-cyanoethylpeptide are stable. The free amino acids remaining in the hydrolysate can be determined by any suitably sensitive method. Since extreme accuracy is not essential, methods involving separation on paper may be used, and a hydrolysate of the unmodified peptide can be run at the same time if required. The only side reactions appear to be the conversion of non-*N*-terminal lysine and cysteine into N^{ϵ}-carboxyethyl-lysine and *S*-carboxyethylcysteine (Weil & Seibles, 1961; Friedman, Cavins & Wall, 1965) respectively.

To the dry peptide (0·005–0·025 μmole) in a soda-glass tube (2 in. × $\frac{1}{4}$ in.) were added 0·1 M-triethylamine (20 μl.) and acrylonitrile (5 μl.). The tube was sealed and the reaction allowed to proceed overnight at 37°. The tube was opened (an ampoule file is convenient) and the contents were evaporated to dryness *in vacuo*. To the residue were added 6 N-HCl (30 μl.) and, if the peptide contained tyrosine, 0·1 M-phenol (3 μl.) (Sanger & Thompson, 1963). The tube was re-sealed and heated overnight at 105°. The tube was opened and the contents were evaporated to dryness *in vacuo* over KOH. The amino acids in the hydrolysate of the *N*-cyanoethylpeptide were determined by the quantitative micro-methods of Blackburn & Lee (1963) (high-voltage paper electrophoresis) or Corfield & Simpson (1965) (high-voltage paper electrophoresis followed by paper chromatography), both of which are based on the measurement of the colour produced when the amino acids on the paper are allowed to react with cadmium–ninhydrin reagent (Heilman, Barollier & Watzke, 1957). The spot corresponding to an amino acid originally present in the *N*-terminal position but not elsewhere in the peptide was decreased to a negligible intensity, or, for certain amino acids (notably glutamic acid, serine and glycine), to the faint background level that is difficult to eliminate completely when working with peptides eluted from paper strips. When the *N*-terminal amino acid was present elsewhere in the peptide, the expected decrease in the intensity of the corresponding spot was observed.

The method requires a minimum of manipulation, and is well adapted for dealing with large numbers of samples. It can be applied to a portion of the residual peptide after one cycle of the Edman degradation to identify a second amino acid residue. The method has been tested on peptides containing up to seven amino acid residues. Some were synthetic di- and tri-peptides, and the others were peptides encountered during sequence work on protein fractions isolated from oxidized wool.

No peptides containing *N*-terminal arginine, lysine, cystine or cysteine were included.

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The Hydrolysis of Unimolecular Films of ^{32}P-Labelled Lecithin, Phosphatidylethanolamine and Phosphatidylinositol with Phospholipase A (*Naja naja* Venom)

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Previous studies on the hydrolysis of unimolecular films of lecithin by venom phospholipase A (EC 3.1.1.4) used changes in the surface potential to measure the extent of the breakdown (Hughes, 1935*a*; Colacicco & Rapport, 1965). It was assumed that the lysolecithin formed remained in the film and that the replacement of lecithin by lysolecithin led to a decrease in the surface potential corresponding to the known differences between the surface potentials of films of pure lecithin and lysolecithin (Hughes, 1935*b*).

The present experiments, with a surface radioactivity technique (Bangham & Dawson, 1960), show that when unimolecular films of three ^{32}P-labelled phospholipids are treated with cobra (*Naja naja*) venom a considerable proportion of the lyso-compound formed actually leaves the film. This loss allows the physicochemical and biochemical determinants of the reaction to be investigated more directly.

The apparatus consisted of a cylindrical glass dish (9cm. diam., 4·5cm. deep) with a central glass-plate barrier (1cm. deep) fused across the diameter of the open end and which divided the surface of the aqueous contents into two semicircular areas. The vessel was nearly filled with 0·9% NaCl solution (275ml.) in 0·73mM tris–HCl buffer, pH 7·2, and a solution of the radioactive phospholipid (20μg. of P/ml.) in $CHCl_3$–ether (1:4, v/v) inserted on the surface of one semicircular area with a microsyringe and the solvent allowed to evaporate. The [^{32}P]lecithin and [^{32}P]-phosphatidylethanolamine were isolated from ^{32}P-labelled *Saccharomyces cerevisiae* (Dawson & Bangham, 1959; Dawson, 1963. [^{32}P]Phosphatidylinositol was isolated from ^{32}P-labelled *Lipomyces lipofer* by using chromatography on formaldehyde-treated paper (Hörhammer, Wagner & Richter, 1959) for the final purification. The surface radioactivity was measured with a mica-window Geiger–Müller counter coupled to a rate-meter and pen recorder. The surface pressure was recorded with a Wilhelmy (1863) dipping plate and torsion balance. Reagents and venom were inserted into the other side of the vessel and the whole contents were stirred magnetically from the underside.

Fig. 1(*a*) shows a typical result of adding 1mg. of venom (0·00036%) under a unimolecular film of [^{32}P]lecithin at a pressure of about 30 dynes/cm. ^{32}P was rapidly lost from the film, the surface

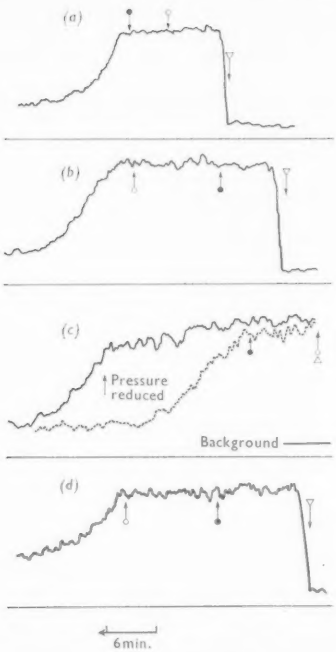

Fig. 1. Effect on the surface radioactivity of unimolecular films of ^{32}P-labelled phospholipids when cobra venom is added underneath. The ordinates represent the surface radioactivity in arbitrary units. The bulk phase consisted of 275ml. of 0·145M-NaCl in 0·73mM-tris–HCl buffer, pH 7·2. ▽, [^{32}P]Phospholipid film formed; ○, 0·73mM-$CaCl_2$ added; ●, 1mg. of cobra venom added. (*a*) and (*b*) [^{32}P]Lecithin film at a surface pressure of 30dynes/cm. (*c*) ——, [^{32}P]Lecithin at a surface pressure of 42·8dynes/cm. when venom was added; pressure was decreased to 21·2dynes/cm. by sucking away part of the film. – – –, [^{32}P]Lecithin film containing 15% (molar) dicetylphosphoric acid at a surface pressure of 44·2 dynes/cm. when venom was added. (*d*) [^{32}P]Phosphatidylethanolamine at a surface pressure of 38·1dynes/cm.

radioactivity declining to a stable level which was some 15–20% of the original. The possibility was considered that this loss was due to the presence in the venom of a phospholipase B (Doery & Pearson, 1964) that was converting the lysolecithin formed into water-soluble glyceryl[^{32}P]phosphorylcholine. However, when the radioactive component was isolated after a film had been digested to completion it all behaved on solvent partition and thin-layer chromatography as lysolecithin. This suggests therefore that at the completion of the reaction the lysolecithin formed is in equilibrium, with about 80–85% in the bulk aqueous phase and the remainder in the film.

The requirement for Ca^{2+} (0·73mM) was absolute (Fig. 1*b*) whatever the surface pressure of the film. It could not be replaced by adding stearylamine to the film or Mg^{2+} to the bulk phase: indeed, the addition of Mg^{2+} antagonized the action of Ca^{2+}. EDTA (1·1mM) completely inhibited the hydrolysis even when the reaction had started.

With films of lecithin at a surface pressure near the collapse (46dynes/cm.) the loss of radioactivity on adding Ca^{2+} and venom was minimal (Fig. 1*c*). When the pressure was gradually lowered a rapid loss of radioactivity began at a pressure of approx. 29–33dynes/cm. Below this pressure the initial rate of reaction (time for half substrate disappearance) appeared to be not appreciably affected by the pressure of the film. Addition of 15% dicetylphosphoric acid (but not stearylamine) to the lecithin caused a greatly increased loss of ^{32}P from high-pressure films in the presence of venom (Fig. 1*c*). However, as the pressure fell an accelerated rate of loss again became apparent at about 30 dynes/cm.

The behaviour of [^{32}P]phosphatidylethanolamine and [^{32}P]phosphatidylinositol films was similar to that of lecithin in that Ca^{2+} was obligatory for the enzymic hydrolysis. Whereas the loss of radioactivity from high-pressure phosphatidylinositol films was very slow, that from similar films of phosphatidylethanolamine was rapid even at high pressures (Fig. 1*d*).

If it is assumed that the slow loss of radioactivity from high-pressure lecithin films is due to a low rate of hydrolysis rather than an inability of the lysolecithin to leave the film, the present results suggest that yeast lecithin in a unimolecular film is not readily attacked by *Naja naja* venom unless the area/molecule is greater than about 75Å^2. The ability of dicetylphosphoric acid to enhance hydrolysis at film pressures above this may be due to the surface dilution caused by the repulsion of the anionic groups introduced into the film and the consequent increased spacing of the lecithin molecules (cf. Bangham & Dawson, 1960). However, it must be emphasized that a similar enhanced hydrolysis is not produced when cationic groups (stearylamine) are introduced into the lipid–water interface. Rather surprisingly, dicetylphosphoric acid does not stimulate the hydrolysis of lecithin particles by cobra venom and indeed acts as a potent inhibitor when the system has been activated with diethyl ether (Dawson, 1963). The hydrolysis of high-pressure films of phosphatidylethanolamine is probably due to these possessing a negative ζ potential owing to there being only partial ionization of the phospholipids' amino groups at the pH used (7·2). It was noted previously (Dawson, 1963) that

large phosphatidylethanolamine particles are hydrolysed without the necessity for the addition of diethyl ether. This latter solvent probably activates the hydrolysis of lecithin particles by causing a wider spacing of the phospholipid molecules orientated at the lipid–water interface as well as preventing the accumulation of the liberated fatty acids at this interface. The lack of hydrolysis of high-pressure phosphatidylinositol films is not consistent with this idea, but here other factors may be operative. Thus such films have a much higher affinity for ^{45}Ca than either lecithin or phosphatidylethanolamine (Dawson, 1965, and unpublished work), and this may have the effect of masking the stimulating effect of the interfacial anionic groupings.

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Heat-Sensitive Deoxyribonuclease Activity in Cells Infected with Herpes Simplex Virus

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The activity of deoxyribonuclease (EC 3.1.4.5) from mammalian cells (BHK 21–C 13) grown in tissue culture increases markedly after infection of the cells with herpes simplex virus (Keir & Gold, 1963). The increment could be ascribed to a stimulation or to a raised level of synthesis of the host-cell enzyme, induced by virus infection. It is also possible that the increment could arise from synthesis of new deoxyribonuclease, directed by the invading viral DNA, and this possibility is consistent with the observation that herpes-virus-specific RNA is transcribed from the herpes DNA after infection of the cells (Hay, Köteles, Keir & Subak-Sharpe, 1966). We now report briefly some observations which indicate that the increase of deoxyribonuclease activity found after infection is attributable to an enzyme(s) that differs in several respects from the corresponding enzyme(s) in the uninfected control cells.

BHK 21–C 13 cells (derived from baby hamster kidney; MacPherson & Stoker, 1962) were grown as described by Russell *et al.* (1964) in 80 oz. bottles. The cell monolayers were infected with herpes simplex virus (strain α) at a multiplicity of exposure 10. At 6–8 hr. later, the cell sheets were washed with phosphate-buffered saline (7·7 mM-Na_2HPO_4–1·5 mM-KH_2PO_4–0·14 M-NaCl) and removed from the glass with 0·5 mM-EDTA in phosphate-buffered saline. The cells were then washed twice with phosphate-buffered saline by centrifugation at 0°, and were stored at −75° until required. After the cells had been thawed, an initial enzyme extraction was carried out essentially as described by Keir & Shepherd (1965), to give a 105 000*g* supernatant fraction (S1) in 0·05 M-tris–HCl buffer (pH 8·0)–0·15 M-KCl–1 mM-EDTA–5 mM-2-mercaptoethanol. The 105 000*g* sediments were suspended in buffer of the same composition, homogenized vigorously for 1 min. in a Potter–Elvehjem homogenizer, and centrifuged at 105 000*g* to give a second soluble enzyme extract (S2). Further re-extraction of the 105 000*g* sediments did not yield significant amounts of enzyme activity.

Deoxyribonuclease was assayed by a standard procedure (Keir, 1962) that measured the release of acid-soluble DNA fragments from native or

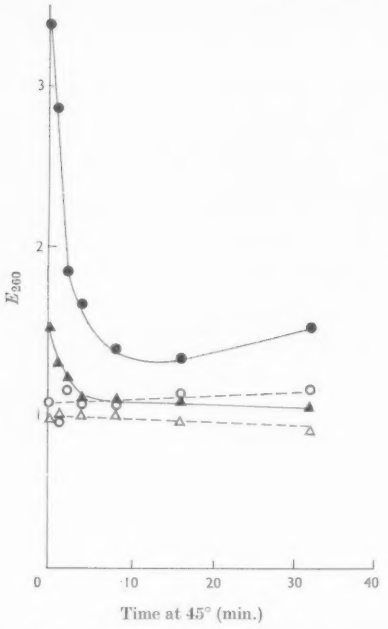

Fig. 1. Heat-stability curves for deoxyribonuclease preparations from uninfected cells (BHK 21–C13) and cells infected for 8hr. with herpes simplex virus. The enzyme fractions were submitted to a prior incubation for the indicated times at 45° and then cooled to 0°. DNA was then added and the mixtures were incubated for 60min. at 37° for assay of surviving deoxyribonuclease activity. Each assay mixture (1·25ml.) contained 25μmoles of tris–HCl buffer, pH 8·0, 75μmoles of KCl, 5μmoles of $MgSO_4$, 0·5μmole of EDTA, 5μmoles of 2-mercaptoethanol, 150μg. of Landschutz ascites-tumour cell DNA (heat-denatured) and 200μg. of protein from the uninfected-cell enzyme fractions (180μg. of protein from the infected-cell fractions). Control samples were run in parallel with the tests (*a*) without added DNA and (*b*) without enzyme. Production of acid-soluble DNA fragments is corrected for these controls and is expressed as E_{260}/mg. of protein. ○, Uninfected-cell S1 preparation; △, uninfected-cell S2 preparation; ●, infected-cell S1 preparation; ▲, infected-cell S2 preparation.

heat-denatured DNA during incubation at 37°. The reaction was linear with respect to release of acid-soluble ultraviolet-absorbing material for at least 90min. When it was necessary to determine the ionic requirements of the reaction, the enzyme preparations were dialysed against 0·05M-tris–HCl buffer (pH 8·0)–1mM-EDTA–5mM-2-mercaptoethanol.

Heat-inactivation experiments. The fractions were submitted to a prior incubation at 45° and pH 8·0 for up to 32min., and were then cooled to 0°. DNA was added, and surviving deoxyribonuclease activity was measured after incubation at 37°. The results (Fig. 1) showed that (*a*) there was substantially more of the nuclease activity in the infected than in the control preparations, (*b*) up to 50% of the nuclease activity in the infected preparations was destroyed by heating for 10min. at 45°, (*c*) the control preparations were stable to the prior heating, and (*d*) though the control S1 and S2 preparations had closely similar activities, the infected S1 preparation was markedly more active than the infected S2 preparation. Since the same amounts of control and infected cells were used, and since in each case the amount of soluble protein in the S2 fraction was about 25% of that in the S1 fraction, it can be concluded that under the extraction conditions used the bulk of the nuclease increment in the infected cells was very readily soluble and extractable. It is perhaps significant that not all of the activity of the infected samples was heat-sensitive; this suggests that the infected cells contain not only the heat-sensitive deoxyribonuclease but also the heat-resistant complement of deoxyribonuclease found in the control cells. When the prior incubation was performed at 50°, the nuclease activity of the infected S1 fraction fell to that of the control S1 fraction within 2min.; thereafter there was a slow decline of activity (25% in 30min.) that closely followed the slow loss of activity in the control preparations.

These heat-inactivation results for control and infected preparations have been observed also with HEp–2 cells (human epitheleoid carcinoma). This is of some importance since the genetic origins of BHK 21–C13 and HEp–2 cells are quite different, and the same virus stock was used in all experiments.

Physical condition of substrate. Though both the control and the infected preparations degrade heat-denatured DNA at a faster rate than native DNA, the increase of deoxyribonuclease activity that follows infection is much greater when related to native DNA as substrate than when related to denatured DNA. However, these relationships may be altered by varying the concentration of Na^+ or K^+ in the assay mixture.

Response to Na^+ *and* K^+. The effects of these cations were tested over the range 0–150mM in the presence of Mg^{2+} at 2mM and tris–HCl buffer (pH 8·9) at 50mM: (*a*) the control activity towards native DNA was little affected, (*b*) the control activity towards denatured DNA and the infected-preparation activity towards native DNA were strongly inhibited by increasing the concentration of either Na^+ or K^+, and (*c*) the infected-preparation activity towards denatured DNA showed an optimum at 60mM-Na^+ or -K^+ (representing a 30%

stimulation over the activity in the absence of Na^+ or K^+).

Requirement for a bivalent cation. All control and infected preparations displayed an absolute requirement for a bivalent cation. Mg^{2+} promoted optimum activity at 2mM, inhibited only moderately at 20mM and showed essentially the same pattern for control and infected cell fractions. Mn^{2+} promoted optimum activity at 0·5mM and inhibited nuclease activity sharply above that concentration, especially with denatured DNA as substrate. Though the activities of the control preparations were the same at 2mM-Mg^{2+} or at 0·5mM-Mn^{2+}, the activities of the infected preparations were only half as great with Mn^{2+} as with Mg^{2+}.

Optimum pH. The effect of pH over the range 6·2–10·5 was studied. With denatured DNA as substrate, the control preparations were more active at pH 6–7 than the infected preparations, although both showed a sharp optimum at pH 9·1. With native DNA as substrate, the infected preparations showed an extended optimum at pH 9–10.

General considerations. From these observations it seems reasonable to conclude that the infected cells contain deoxyribonuclease activity that is not present in the control uninfected cells. There is no clear indication whether production of the enzyme(s) is programmed by the viral or by the host-cell DNA. It would seem that this point could be conclusively proved only by syntheses *in vitro* of enzyme directed by the respective DNA. Similar considerations apply also to DNA nucleotidyltransferase (EC 2.7.7.7), some of the properties of which are altered after infection of BHK 21–C 13 cells of HEp–2 cells with herpes simplex virus (Keir, Hay, Morrison & Subak-Sharpe, 1966).

Satisfactory characterization of the deoxyribonuclease activities described here clearly depends on their purification. Preliminary experiments on purification have indicated that the control-cell and infected-cell nuclease activities behave differently during chromatography on DEAE-cellulose.

We thank Professor J. N. Davidson, F.R.S., for his interest and support, and Dr H. Subak-Sharpe for his kindness in providing the cells and virus. The investigation was aided by a grant from the British Empire Cancer Campaign. J. M. M. is a Medical Research Council Scholar.

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **454th Meeting** *of the Society was held in the Chemistry Department, Manchester University on Thursday and Friday,* 11–12 *November,* 1965, *when the following papers were presented:*

COLLOQUIUM ON 'BIOSYNTHESIS IN MICRO-ORGANISMS: GENETIC AND ENVIRONMENTAL FACTORS'

Introductory Remarks and Comments on the Interactions between Environmental and Mutational Effects in Yeasts

By G. R. Barker. (*Department of Biological Chemistry, The University, Manchester,* 13)

Saccharomyces cerevisiae strain 285A was shown by Abrams (1952) to require either adenine or hypoxanthine for growth and to develop a pink colour on exhaustion of these bases in the medium. It was also reported that adenine suppresses *de novo* synthesis of nucleotides in wild strain yeast, but stimulates this synthetic route in the mutant 285A. Mr S. R. Ayad has investigated this mutant in these laboratories. The reported effect of adenine on *de novo* nucleotide synthesis was not confirmed, and, in addition, it was found that the purine requirement could not be fulfilled by guanine or xanthine, suggesting a complex metabolic deficiency.

Using cell-free extracts, it was found that the mutant strain is unable to synthesize nucleotides from isotopically-labelled glycine, indicating a block in the *de novo* synthesis of nucleotides. In addition, it was found that formation of inosine 5′-phosphate from labelled guanine or xanthine did not take place. Phosphoribosyl-aminoimidazole-carboxamide formyltransferase (EC 2.1.2.3), IMP-cyclohydrolase (EC 3.5.4.10) and GMP-reductase (EC 1.6.68) were purified from cell-free extracts of the wild strain and it was shown that corresponding fractions from the mutant contained little or no enzymic activity. These results will be discussed in relation to the nature of the mutation and the environment required for expression of the phenotype.

A spontaneous mutation in *Candida utilis* strain IMI 23311 (now designated NCYC 321) has been examined in these laboratories by Mrs Vendulka Jechová (Barker & Jechová, 1964). This mutation involves at least one block in the pre-formed purine route of nucleotide synthesis and, in terms of nucleotide metabolism, is complementary in nature to the mutation in *S. cerevisiae* discussed above. Since it may be presumed that *de novo* synthesis is the principal synthetic route to purine nucleotides, this type of mutant would not be selected by normal procedures. Its behaviour under different nutritional conditions and in mixed populations of cells will be discussed.

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Extrachromosomal Genes and Enzyme Synthesis in Bacteria

By M. H. Richmond. (*Department of Molecular Biology, University of Edinburgh, King's Buildings Site, West Mains Road, Edinburgh,* 9)

It has been realized for many years that the DNA of animal and plant cells is carried as a number of separate pieces, or chromosomes, and that the gene content of the cell is distributed among them. The fragmentation of DNA was also commonly seen in protozoa but until relatively recently it was thought that all bacterial genes were present on a single chromosome. The discovery of the F (Sex, or Fertility) factor (Hayes, 1953) and the demonstration that this particle contained DNA and could be carried in an extrachromosomal state by the bacterial cell showed that all the DNA of the bacterium was not necessarily part of a single chromosome. Moreover, under appropriate conditions the F-particle is capable of incorporating certain 'chromosomal' genes thus taking them into the extrachromosomal state and also transferring them to a recipient cell which may, or may not, already possess them.

The discovery that bacterial genes can be carried in an extrachromosomal state and can be transferred from cell to cell at high frequency has two far-reaching implications for bacterial enzyme synthesis, in the broadest sense. First, it allows the transfer of a number of genes at once from cell to cell. A recipient cell can, therefore, gain the ability to make a number of enzymes (for example, the component enzymes of a biosynthetic pathway) at once, and this has already been shown to be of

great importance in the adaptation of bacterial *populations* to a change in environment.

The second implication concerns possible routes for the evolution of enzymes, and is more speculative. One great difficulty in basing the evolution of a protein on a process of step-wise modification by mutation is that the first mutation may cause a loss of enzyme activity and thus be lethal to the survival of the organism unless the loss is 'covered' in some way. It is possible that the extrachromosomal state could assist in providing 'cover' for mutations which otherwise would be lethal and thus play a vital part in enzyme evolution.

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Development of Secondary Biosynthesis in Moulds

By J. D. Bu'Lock. (*Department of Chemistry, The University, Manchester*, 13)

Amongst the metabolic activities of micro-organisms, those usually classed as *secondary* are far from trivial: they can contribute significantly to a fuller understanding of microbial biology. Their study has to some extent been discouraged by their apparently meaningless diversity, but in recent years a better understanding of biosynthetic mechanisms has revealed general unities, even in the actual processes of diversification. Equally the fact that secondary metabolic activity is not invariably manifest in an organism is now seen as an essential ingredient of our understanding.

Viewed as a biochemical manifestation of differentiation during development, the phenomena of secondary metabolism pose new questions in terms of metabolic regulation and gene expression. Some studies of typical cases have been made in such specific terms, but most of the evidence so far available must be derived indirectly. This can be illustrated by interpretations of several well-known observations, and by some studies of my own colleagues, notably Dr A. J. Powell, Mr M. A. Hulme, and Mr D. Shepherd.

For example, it will be shown that the metabolic crisis which occurs at the onset of secondary metabolism in *Penicillium urticae* (Bu'Lock, Hamilton & Powell, 1962; Bu'Lock *et al.* 1965) is directly connected with changes in the tricarboxylic acid cycle which reflect changes in the utilization of intermediates for amino acid synthesis. Moreover, radiorespirometric studies of the kind already described for *P. urticae* have now confirmed essentially similar phenomena in, for example, *Gibberella fujikuroi*, *Claviceps purpurea*, and *Penicillium islandicum*, despite the superficially dissimilar patterns of secondary metabolism in these species.

In *P. urticae* we have also investigated further, as an example of regulation mechanisms, the effects of the parent phenol, 6-methylsalicylate, on the synthesis of ultimate products such as patulin; these effects were first observed, though differently interpreted, by Bassett & Tanenbaum (1960). We find that although 6-methylsalicylate is the precursor of patulin it can inhibit the synthesis of patulin in two ways. The first involves the general inhibitory effect of salicylates and in particular an inhibition of the uptake of exogenous substrates. The second is a specific effect, observable at lower concentrations, resulting from "feedback" inhibition of the synthesis of 6-methylsalicylate itself. In addition the organism has a mechanism for regulating the uptake of 6-methylsalicylate from the medium; added 6-methylsalicylate is rapidly taken up at first, but after a period varying from a few min. to 30 min. the added phenol is actively expelled. The re-appearance is not due to any *de novo* synthesis since labelled 6-methylsalicylate is not correspondingly diluted.

We are grateful to the D.S.I.R. for grants in aid of our work, to colleagues in many parts of the world for discussion, and to Professor A. J. Birch for his support.

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The 'Point of No-Return' in Cell Differentiation in *Blastocladiella*

By E. C. Cantino. (*Department of Botany and Plant Pathology, Michigan State University, East Lansing, U.S.A.*)

In 1949, we discovered, isolated in pure culture, and later named as a new species, the aquatic Phycomycete (water mould) known as *Blastocladiella emersonii*. This two-celled fungus was capable of developing along either of two major pathways: in the presence of exogenous bicarbonate, it formed a terminal, thick-walled, brown, RS cell, and in its absence, a thin-walled, colourless, OC cell. It is currently believed that the focal point for this morphogenetic effect lies in a bicarbonate-induced NADPH-specific reductive carboxylation of oxoglutarate to isocitrate and the concomitant removal of the latter *via* sequential reactions mediated initially by isocitratase and glycine-alanine transaminase.

An attempt will be made to summarize our first 15 years of evidence for the nature of this bicar-

bonate trigger mechanism (Cantino & Lovett, 1964; Cantino, 1966).

Subsequentially, attention will be focused on work in progress dealing with the way in which reducing power (NADPH), needed for the reductive carboxylation of oxoglutarate, is generated *via* glucose 6-phosphate dehydrogenase (G6PDH) as the RS cell approaches, reaches, and passes its morphological point of no return. Specifically, this involves (*a*), *in vitro* (Pandhi & Cantino, unpublished results) and *in vivo* (Cantino & Pandhi, unpublished results) evidence for alterations in isozymic composition and intracellular distribution of G6PDH, and (*b*), tracer studies (Cantino & Pandhi, unpublished results) on the relation between binding and release of glucose by the cell, on the one hand, and the cell's capacity for morphological reversal, on the other.

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Physiological and Metabolic Differentiation in Micro-organisms

By I. Malek. (*Czechoslovak Academy of Science, Institute of Microbiology, Praha* 4, *Czechoslovakia*)

COMMUNICATIONS

The Fractionation of Transforming DNA from *Bacillus subtilis*

By S. R. Ayad, G. R. Barker and A. E. Jacob. (*Department of Biological Chemistry, The University, Manchester*, 13)

Pneumococcal transforming DNA has been fractionated using histone-kieselguhr (Ephrussi-Taylor, 1955), methylated bovine serum albumin-kieselguhr (MAK) (Lerman, 1955) and ECTEOLA resin (Bendich, Pahl & Beiser, 1956). Preliminary experiments in these laboratories with transforming DNA from *B. subtilis* indicated that MAK columns gave the most reproducible results.

Mandell & Hershey (1960) showed that elution of DNA from MAK columns with solutions of increasing salt concentration gave fractions having progressively higher molecular weight.

Saito & Masamune (1964) reported the chromatography of transforming DNA from *B. subtilis* on MAK. Resolution of the material was poor as judged by extinction at 260mμ but evidence was presented that later fractions had suffered less degradation.

In the present study, by using methylated albumin prepared by the method of Hayashi, Hayashi & Spiegelman (1963), and by increasing the layer of absorbent in which fractionation takes place, transforming DNA from *B. subtilis* (Marburg strain) has yielded three partially resolved peaks. Re-chromatography of material from the third peak gave an apparently homogeneous preparation. Assay of different fractions for transforming activity using *B. subtilis* strain 31, which is auxotrophic for histidine and tryptophan, showed that activities for the two markers were proportional to the extinction of the fraction at 260mμ. Assays for transforming activity for the arginine marker, using *B. subtilis* strain 3115, indicated that the DNA having highest transforming activity for this marker is eluted considerably earlier than the DNA active in transformation for the histidine or tryptophan markers. The results suggest that partial separation has been achieved of DNA corresponding to the genes associated with the synthesis of histidine and tryptophan on the one hand, from that corresponding to the genes associated with arginine synthesis on the other hand. These results are in agreement with the genetic evidence that the two former genetic loci are linked together but are not linked to the latter gene (Saito & Masamune, 1964).

The authors are indebted to Dr H. Saito for the gift of cultures. Financial support from the Ministry of Higher Education of Egypt (S. R. A.) and the Medical Research Council (A. E. J.) is gratefully acknowledged.

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Ribosomal Particles of *Pisum arvense*

By G. R. Barker and M. Rieber. (*Department of Biological Chemistry, The University, Manchester*, 13)

It has been shown (Barker & Hollinshead, 1965) that the ribosomes of cotyledons from germinating seed of *Pisum arvense* are more sensitive to low concentrations of magnesium ions than particles from the growing parts of the seedling. This raises the question as to whether hypogeal cotyledons possess an operative protein synthesizing system, or whether the atypical nature of the ribosomes is an artifact. It has now been shown that cell-free extracts of peas taken after one day's germination and, to a less extent, dry peas, are able to incorporate labelled phenylalanine into protein and attempts were therefore made to demonstrate the presence of polysomes in these tissues. In presence of 10mM-Mg^{2+}, both tissues yielded particles having sedimentation coefficients of 110s, 70s and 50s, but no faster sedimenting material. If, however, in addition to the above magnesium ion concentration, the medium is made 5mM with respect to Zn^{2+}, particles having sedimentation coefficients of 170s, 140s, 110s, 70s and 50s were observed in extracts of seed germinated for one day; under these conditions extracts of dry seed gave the same results as in the absence of zinc ions. Experiments have demonstrated that the ribonuclease activity of crude extracts of cotyledons is strongly inhibited by zinc ions, and it is believed that the absence of faster sedimenting particles in preparations made from germinated seed in absence of zinc is due to their degradation by ribonuclease. This explanation, however, cannot be applied to preparations of dry seed. Various factors influencing the preparation of polysomes from dry seed will be discussed, including the availability of magnesium ions and the binding of polysomes to membranes in a manner analogous to that found by Aronson (1965) in bacterial spores.

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Ribonucleases of *Staphylococcus aureus* (strain Duncan)

By G. R. Barker and J. G. Pavlik. (*Department of Biological Chemistry, The University, Manchester*, 13)

Ribonucleases have been recognized in a number of bacteria and are frequently associated with the ribosomal fraction of the disrupted cells. In the present work, it has been found that ribonuclease activity is associated with ribosomal preparations from *Staph. aureus*. Activity is optimal at pH 5·8 in presence of 4M-urea which liberates the enzyme from the ribosomal particles. In addition to this enzyme ribonucleases have been recognized in the cell sap of *Staph. aureus* and in the culture medium and these enzymes have been studied in great detail.

Extracellular enzymes having ribonuclease activity have been reported in cultures of a number of bacteria. The culture medium of the organism referred to by the authors as *Micrococcus pyogenes* (var. *aureus*) has yielded material exhibiting both ribonuclease and deoxyribonuclease (micrococcal nuclease (Cunningham, Catlin & Privat de Garilhe, 1956). Some doubt exists as to whether or not the two activities are associated with one enzyme (Cunningham, 1958; Reddi, 1958). A ribonuclease having optimal activity at pH 7·8 was recognized in the culture medium of *Staph. aureus* (strain Duncan) by Cannon (1961). In the present work, this enzyme has been purified and obtained as a protein homogeneous in the ultracentrifuge and free from deoxyribonuclease activity. Cells of the same organism, after rupture by shaking with glass ballotini, were centrifuged at 100000*g* and yielded a preparation exhibiting ribonuclease activity optimally at pH 7·8 in presence of 10^{-4}M-*p*-chloromercuribenzoate (PCMB). The enzyme was inactive in absence of PCMB and was not activated by the addition of either urea or EDTA. The latent intracellular enzyme was partially purified in presence of PCMB and resembled the extracellular enzyme in its behaviour on precipitation with ammonium sulphate and on partition chromatography. Both enzymes migrate similarly on electrophoresis. The extracellular enzyme is secreted into the medium during the exponential phase of growth and is present in highest concentration at the onset of stationary phase. In view of the close similarity between the two enzymes, it is considered that the latent enzyme in the cell sap is the precursor of the extracellular enzyme and that activation takes place during passage through the cell membrane. The relevance of the extracellular enzyme to the growth of the organism and its nutritional requirement for uracil will be discussed.

Financial support from the British Empire Cancer Campaign is gratefully acknowledged.

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Puromycin-Initiated Release of Soluble Ribonucleic Acid from Ribosomes of *Escherichia coli*

By M. CANNON (introduced by H. R. V. ARNSTEIN). (*National Institute for Medical Research, Mill Hill, London, N.W.* 7)

Ribosomes from *Escherichia coli* have one site on the 50s subunit that can bind an s-RNA molecule in the absence of protein synthesis. This binding is stabilized by 10^{-2}M-Mg^{2+} and lowering of the concentration to 10^{-4}M releases the s-RNA. After protein synthesis in an *E. coli* cell-free extract, nascent polypeptide chains linked terminally to s-RNA molecules are produced on the ribosomes, and these complexes bind strongly to the 50s ribosomal subunits and are stable in 10^{-4}M-Mg^{2+} (Cannon, Krug & Gilbert, 1963).

An attempt has been made to determine if the s-RNA linked to the growing polypeptide chain is bound to the same site identified in the absence of synthesis. Use has been made of puromycin, which releases nascent protein from ribosomes by breaking the s-RNA-polypeptide bonds (Morris & Schweet, 1961). In the absence of further protein synthesis, this mechanism will expose free s-RNA molecules on the ribosome and their stability may be investigated.

E. coli crude extracts were labelled with [^{14}C]-*Chlorella* protein hydrolysate and then chilled to 0°. Puromycin (10^{-4}M) was added, and after 30min. at 0° the amount of nascent protein on the ribosomes was determined by sucrose gradient centrifugation in 10^{-4}M-Mg^{2+}. Under these conditions puromycin releases about 50% of the ribosomal-bound protein.

Similar incorporations were carried out in the presence of [^{32}P]s-RNA. After puromycin stripping at 0°, s-RNA previously bound to nascent protein dissociates from the ribosomes in 10^{-4}M-Mg^{2+}. In this respect, therefore, the s-RNA-polypeptide binding site is indistinguishable from the site identified in the absence of synthesis. The normal stability of the former site results, presumably, from the presence of the polypeptide.

Puromycin added to a crude extract at 30° removes up to 80% nascent protein during 10min. incubation. Under these conditions ribosomes contain very short peptide chains which are constantly synthesized and then removed by puromycin action. Since these chains terminate in s-RNA molecules, analysis of such reaction mixtures always shows considerable amounts of bound s-RNA not dissociable in 10^{-4}M-Mg^{2+}. Only very short peptide chains are necessary for stable s-RNA binding.

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Histones from Rat Liver and Thymus

By MARGERY G. ORD and L. A. STOCKEN. (*Department of Biochemistry, University of Oxford*)

Nuclei were prepared from liver and thymus glands of rats previously injected with [^{14}C]adenine, [^{14}C] amino acid mixture or $^{32}PO_4$. Histones were obtained either by methods I and II of Johns (1964) or by extraction with 50 and 250mM-HCl (Ui, 1957). The 50mM-HCl extract was brought to 1·25N in HCl, 4vol. of ethanol added and the solution dialysed against ethanol. The precipitate was redissolved in 50mM-HCl, $HClO_4$ added to 0·74N and the precipitate suspended in 50mM-HCl and dialysed against the same concentration of acid to remove $HClO_4$. This process removed f1 and $f2_a$ and the remaining solution contained phosphate and thiol groups in approximately equal proportions. Fractions $f2_a$ and $f2_b$ prepared by method II of Johns (1964) had negligible phosphate or thiol contents. Fraction f1 was shown to contain one phosphoserine per mol.wt. of 42–43000 and had a low rate of amino acid incorporation. In agreement with Phillips (1965) insignificant SH or SS was present.

Exclusion chromatography on Sephadex G-75 followed by electrophoretic analysis on acrylamide gel (McAllister, Wan & Irvin, 1963) showed the presence of protein(s), having higher amino acid uptake than the major components. Thiol groups and phosphate were associated with the rapidly labelled protein. The uptake of [^{14}C]adenine suggested that the phosphate might be present as nucleotide.

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The Histones of Trout Liver

By J. PALAU* and J. A. V. BUTLER. (*Chester Beatty Research Institute, Institute of Cancer Research: Royal Cancer Hospital, Fulham Road, London, S.W.* 3)

The histones of a number of mammalian species and organs have been examined and it has been

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found that the whole group of histones and the individual members of the groups are remarkably alike (Hnilica, Johns & Butler, 1962; Laurence, Simson & Butler, 1963; Laurence & Butler, 1965; Laurence, Phillips & Butler, 1965) although plant histones have been found to differ (Johns & Butler, 1962). It therefore seemed desirable to examine the histones of a species of a phylum present earlier than mammals in the evolutionary sequence and a study has been made of the histones of trout liver.

The amino acid composition, *N*-terminal groups and starch-gel electrophoresis pattern of the whole histone extracted from trout liver nuclei, isolated by the methods reported by Laurence & Butler (1965) are similar to those of calf thymus. The following fractions were obtained by methods similar to those used for the fractionation of the histones of calf thymus (Johns, 1964, Phillips & Johns, 1965): F1, F2a1, F2a2, F2b, F3. In each case these were closely similar in composition, *N*-terminal groups and starch-gel pictures with the corresponding fractions of calf thymus histone. It therefore appears that the whole set of histones present in mammalian tissues is present in species existing prior to the emergence of mammals. This does not exclude the possibility of minor differences of amino acid sequence in these proteins.

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Origin of Natural Acetylenes

By J. D. Bu'Lock and G. N. Smith.* (*Department of Chemistry, The University, Manchester*, 13)

We have recently put forward hypotheses for the biogenesis of natural acetylene derivatives, (Bu'Lock, 1965). Briefly, it was suggested that common, taxonomically appropriate, fatty acids were the eventual precursors, into which double and triple bonds were introduced successively by specific dehydrogenations, or some equivalent mechanism, with reactions of chain-shortening (e.g. by β-oxidation) and of allylic and propargylic rearrangements (e.g. *via* allenes) to give eventually the shorter-chain multiply-unsaturated acids which are known to be key intermediates for the diversification phases of polyacetylene biosynthesis. Monoacetylenic derivatives of the common fatty acids are critically important in such a scheme.

For example, the wide range of polyacetylenes obtainable from higher fungi (Jones, 1965; Jones, Lowe & Lowe, 1964*a*,*b*) are in this hypothesis all derived from oleic acid *via* linoleic (predominant in fungal lipids), with crepenynic acid (octadec-9-en-12-ynoic, i.e. 12,13-dehydrolinoleic) as the first acetylenic intermediate. We now find that in the fungus *Tricholoma grammopodium*, producing typical C_{10} polyacetylenes, a substance constituting approx. 0·5% of the lipids is identifiable as crepenynic acid by its vapour-phase and thin-layer chromatographic behaviour and by oxidative degradation; previously (Mikolajczak, Smith, Bagby & Wolff, 1964), this acid was known only from a higher plant species in that section of Compositae (Liguliflorae) which does not produce typical polyacetylenes. In addition, a trace component of our samples, chromatographically distinct, gave butyric acid on oxidation, and from mass spectra of the ketone derived from its methyl ester, this is identified as octadec-9,14-dien-12-ynoic acid, i.e. 14,15-dehydrocrepenynic, which represents the next stage in the biosynthetic sequence leading eventually to typical C_{10} polyacetylenes.

In the same organism, added [10-^{14}C]oleic acid is converted into linoleic and crepenynic acids which are labelled only in the $C_{(9)}$–$C_{(11)}$ region, presumably at $C_{(10)}$, and simultaneously into the typical polyacetylene dehydromatricarianol (dec-2-en-4,6,8-triyn-1-ol) which is specifically labelled at $C_{(2)}$ as expected. Thus all essentials of our hypothetical scheme are verified: (i) conversion of ethylenic to acetylenic groups, (ii) further desaturation of acetylenes at adjacent centres, (iii) formation of shorter-chain polyacetylenes from simple longer-chain fatty acids. Since pathways of this kind apply equally well to the whole range of acetylenic products from higher plants, the biogenetic and chemotaxonomic implications are considerable; the actual mechanisms of desaturation remain open to investigation.

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Aminoacetone Production by Micro-organisms

By J. M. TURNER. (*Department of Biochemistry, The University of Liverpool, Liverpool*, 3)

Whereas aminoacetone formation from L-threonine and from glycine has been demonstrated using washed cell suspensions of *Staphylococcus aureus* (Elliott, 1958, 1959, 1960) and *Rhodopseudomonas spheroides* (Neuberger & Tait, 1960, 1962), no systematic survey of the ability of micro-organisms to produce aminoacetone has been reported. The preliminary results of such a survey, using a variety of possible precursors of the amino-ketone, reveal that the ability is widespread. Threonine was found to be most effective precursor in the cases of *Aeromonas liquefaciens*, *Sarcina lutea*, and *Bacillus* species, but in contrast DL-1-aminopropan-2-ol was more effective using *Pseudomonas aeruginosa*, *Agrobacterium tumefaciens*, *Achromobacter anitratum*, *Escherichia coli*, *Erwinia carotovora*, *Proteus vulgaris*, *Staphylococcus afermentans*, and *Streptomyces griseus*. Aminoacetone production from glycine, plus acetate or pyruvate, was relatively poor in all cases studied. Evidence that DL-4-amino-3-hydroxybutyrate may act as a precursor of aminoacetone has been obtained with a number of species, the most active of which was *Pseudomonas aeruginosa*. All organisms studied were grown on complex natural media. Preliminary results with closely related species of Pseudomonads suggest that aminoacetone production from various precursors could serve as a metabolic characteristic useful for the differentiation of such micro-organisms.

The formation of aminoacetone from aminopropanol has not been reported previously and was examined in detail using *E. coli*. With washed cells, the optimum pH was 6·4, the apparent K_m for aminopropanol was 0·3mM with respect to single stereoisomers, and no metal ion requirements were found. Cell-free extracts exhibited 1-aminopropan-2-ol dehydrogenase activity optimally at pH 7, the K_m for the substrate was 0·8mM, K^+ ions were required, and NAD was the most effective coenzyme tested. Although extracts of *E. coli* also exhibited L-threonine dehydrogenase activity (Neuberger & Tait, 1962; Green & Elliott, 1964), the enzymes were found to be distinct on the basis of kinetic data and the repressibility of aminopropanol dehydrogenase by glucose and other compounds. Although the stereospecificity of the reaction has not yet been established, it appears likely that the conversion of aminopropanol to aminoacetone by an oxidoreductase may be involved in the biosynthesis of the D-1-aminopropan-2-ol moiety of vitamin B_{12} from L-threonine (Krasna, Rosenblum & Sprinson, 1957).

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Di-Ligand Metal Ion Buffers: the Binding of Cupric Ions by Ribonuclease

By R. H. SAUNDRY and W. D. STEIN. (*Department of Biological Chemistry, The University, Manchester*, 13)

Metal ion buffers previously proposed for biological investigations have used the formation of one-to-one complexes of metal ion and ligand. A limited number only of such mono-ligand complexes are available, but many more ligands form metal–di-ligand complexes. The following equation describes such complex formation and gives the negative logarithm of the free metal ion concentration as:

$$\mathrm{pM} = \log \beta_2 - 2\log(1+\mathrm{H}^+/K_{\mathrm{HL}}) + 2\log(\mathrm{L}_{total}) - \log(\mathrm{M}_{total})$$

where β_2 is the product of the first and second association constants for the metal-ligand complex, L_{total} is the total concentration of ligand present, and M_{total} that of metal. K_{HL} is the second acid dissociation constant of the ligand.

The derivation of this equation assumes that $L_{total} \gg M_{total}$ and that $pH \gg pK_{H_2L}$ for the ligand—thus only K_{HL} need be considered.

The theory was used with the system copper: β-alanine in formulating copper ion buffers for an equilibrium dialysis study of copper ion binding by bovine pancreatic ribonuclease at 25°. Acetate and maleate pH buffers enabled binding experiments to be carried out at pH 6·1 and 7·0 (ionic strength, 0·1).

The copper binding data, handled after the method of Hughes & Klotz (1956), gave $1{\cdot}51 \pm 0{\cdot}27$ copper binding sites per ribonuclease molecule at pH 6·1, while $2{\cdot}31 \pm 0{\cdot}056$ sites were found at pH 7·0. The apparent affinity constants were $3{\cdot}89 \times 10^5$ at pH 6·1 and $5{\cdot}14 \times 10^6$ at pH 7·0.

Comparison of the data at the two pH values, making the approximation that the number of binding sites remained constant, and assuming that only one group in each binding site was dissociating in the pH range studied, lead to a value of 7·2 for the pK_a of the binding site and a value of $10^{6\cdot8}$ for the true affinity constant of each binding site for

copper ions. The data suggest (1) that the ions are chelated to the sites and (2) are consistent with a binding by histidyl residues. Parallel studies of the inhibition of ribonuclease by copper ions at pH 7·0 suggest that one of the first copper ions to be bound binds at the enzyme active centre.

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The β-Galactoside Permease of *Escherichia coli* as a Primary Active Transport System

By J. Kolber and W. D. Stein. (*Department of Biological Chemistry, The University, Manchester*, 13)

The active accumulation of sugars and amino acids by intestine (Czaky, 1965) and of glycine by erythrocytes (Vidaver, 1964) and ascites tumour cells (Eddy & Mulcahy, 1965) appears to be brought about by the mandatory co-transport of sodium ions as the metabolites cross the cell membrane. The free energy for such secondary active transports derives from the free energy inherent in the electrochemical gradient of sodium ions, this gradient being maintained by an ATP-linked primary transport of sodium. To ascertain whether the active transport of β-galactosides by the permease of *Escherichia coli* (Kepes, 1960) is such a sodium-dependent secondary transport system, we have investigated whether there is a mandatory requirement for sodium ions during β-galactoside transport.

The initial rate of uptake of *o*-nitrophenyl-β-D-galactoside (ONPG) by *E. coli* was measured at 0° as a function of the ONPG concentration, in the presence of either 0·1M-sodium chloride or potassium chloride or 0·2M-sucrose. In all three media, ONPG was effectively transported. Reciprocal plots of the data showed that the maximum velocity of ONPG uptake was unaffected by the presence or absence of sodium ions, but that the K_m term was dependent on the ionic composition (in sodium chloride, K_m is 0·6mM; in potassium chloride K_m is 2·0mM; in sucrose K_m is 1·2mM.). Since the value of K_m depends on the local ionic composition, a sodium ion gradient could produce a gradient in ONPG. A kinetic analysis of the relevant transport equations predicts a maximum distribution ratio of ONPG of 3·3, at the prevailing levels of cellular sodium and potassium, and predicts that this ratio should be independent of the ONPG concentration.

In contrast, we find that at 1mM-ONPG, the initial distribution ratio is 11, and this ratio depends on the ONPG concentration. Extrapolating from our results, a maximum distribution ratio of 40 would be expected at zero ONPG concentration.

A small part only of the steady-state accumulation of *o*-nitrophenyl-β-D-galactoside can therefore arise from coupling β-galactoside and sodium transport. The major portion must derive from a primary transport (probably linked to ATP breakdown) or else is coupled to the transport of some unidentified cell constituent.

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Isolation of Angolide from *Pithomyces sacchari* (Speg.) M. B. Ellis

By Pamela Riches and D. W. Russell. (*Twyford Laboratories, Twyford Abbey Road, London, N.W.* 10)

In all species of the fungal genus *Pithomyces* examined, the conidia are covered with crystalline spicules. In those species which sporulate freely, the spicules are known to consist of cyclodepsipeptides. However, *P. sacchari* growing in static liquid culture formed too few conidia for the surface spicules to be isolated, and their chemical nature remained unknown (Bertaud, Morice, Russell & Taylor, 1963; Bishop, Griffiths, Russell, Ward & Gartside, 1965).

Isolate IMI102682 of *P. sacchari* was grown on Ross medium to which 2% (w/v) of agar had been added, under conditions otherwise as before. After 2 weeks, moderate numbers of spores had been formed. The surface of the mature cultures was covered with chloroform, which was rocked gently for 5min., then decanted through siliconed filter paper. Material obtained by evaporating three successive extracts was pooled, and the ether-soluble portion was discarded. The chloroform-soluble portion of the residue was crystallized to constant specific rotation. *Pithomyces* TPI122, kindly sent to us for study by Dr M. O. Moss of the Tropical Products Institute, and disposed in the *P. sacchari* complex (Dr M. B. Ellis, personal communication), similarly gave a crystalline compound.

The physical properties of these two products were identical with those of the cyclotetradepsipeptide angolide (Russell, 1965), previously isolated from *P. cynodontis* IMI101184 (Ellis, 1965). Some danger of ambiguity attends the identification of cyclodepsipeptides by their physical properties alone (see, e.g. Shemyakin, Ovchinnikov, Ivanov & Kiryushkin, 1963; Bertaud, Probine, Shannon & Taylor, 1965). Acid hydrolysates were therefore examined by paper chromatography; they contained in both cases isoleucine and *allo*isoleucine (1:1) and α-hydroxyisovaleric acid. Mild hydrazinolysis of either sample gave two hydrazides,

respectively indistinguishable by chromatography or electrophoresis from the hydrazides of L-α-hydroxyisovaleryl-L-isoleucine and L-α-hydroxyisovaleryl-D-*allo*isoleucine, which are formed by hydrazinolysis of angolide (Russell, 1965).

The ready accessibility to chloroform of angolide in *P. sacchari*, and the analogy with other species, suggest that it forms the conidial spicules of this species.

On the basis of this and previous work the genus *Pithomyces* may be divided into two groups. The first comprises *P. chartarum* and *P. maydicus*, which produce 'irregular' cyclohexadepsipeptides (sporidesmolides). The second contains *P. cynodontis* and members of the *P. sacchari* complex, which includes 'species V' (Dr M. B. Ellis, personal communication). These produce the cyclotetradepsipeptide angolide.

We thank Dr V. T. Ivanov for optical rotatory dispersion measurements.

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Changes in the Regional Lymph Nodes of Specific-Pathogen-Free Rats after Injection of Dead Tubercle Bacilli Suspended in Mineral Oil into the Hind Foot Pad

By T. J. Franklin. (*Imperial Chemical Industries Limited, Pharmaceuticals Division, Alderley Park, Cheshire*)

Rats are raised in the Alderley Park colony under specific-pathogen-free conditions. The predominant cell in the lymph nodes of these animals is the small lymphocyte. There are very few blast cells or plasma cells, which in contrast with the small lymphocytes have abundant cytoplasm containing many ribosomes. Sucrose density-gradient analysis of disrupted cell preparations from these lymph nodes revealed 80s and 120s ribosomes but little polyribosomal material.

When a suspension of dead tubercle bacilli in liquid paraffin is injected into the hind foot pad, there is a rapid increase in the size of the regional lymph nodes beginning at about 48hr. and reaching a maximum between 4 and 5 days after the injection. Many blast cells appear in the nodes and also smaller numbers of plasma cells. Cell suspensions prepared from the nodes of normal rats and at intervals after the injection of the tubercle suspension showed a progressive increase in the rate of uptake of labelled protein and nucleic precursors *in vitro*, reaching a maximum between 3 to 4 days after injection. Sucrose density-gradient analysis of disrupted cell preparations from the nodes 4 days after injection indicated a marked increase in the area corresponding to polyribosomes with sharp peaks at 270s, 205s and 175s. Treatment of the cell homogenates with ribonuclease (0·5μg./ml.) at 0° for 30min. converted the polyribosomal material to 80s and 120s ribosomes.

Inhibition of Ram-Testis Glycosidases by Acid Mucopolysaccharides

By J. C. Caygill. (*Rheumatism Research Centre, University of Manchester, Clinical Sciences Building, York Place, Manchester*, 13)

To measure β-*N*-acetylglucosaminidase activity in the synovial fluid (Caygill & Jevons, 1965) of rheumatoid arthritis patients, it is necessary to remove leucocytes by high speed centrifugation. However, when the apparently cell-free fluid was ultracentrifuged, the free activity increased. Ultracentrifugation may remove hyaluronic acid, or some other high molecular weight inhibitor.

Experiments showed that ram testis β-*N*-acetylglucosaminidase (EC 3.2.1.30) was competitively inhibited by hyaluronic acid at concentrations of 10–100μg./ml., whilst β-galactosidase (EC 3.2.1.21) and β-glucuronidase (EC 3.2.1.31) were non-competitively inhibited. The inhibition of each enzyme was greatest in acid solutions, and was not detectable in neutral solutions. Varying the concentration of enzyme in the presence of a fixed quantity of hyaluronic acid revealed a greater percentage inhibition at low enzyme concentration than at higher enzyme concentrations. At pH 4·3, increasing the concentration of hyaluronic acid gave a curve of β-*N*-acetylglucosaminidase activity which fell sharply at low hyaluronic acid concentrations, but a less steep fall at higher concentrations. All three glycosidases were non-competitively inhibited by heparin and by chondroitin sulphate. The inhibition could be reversed by the addition of calcium chloride or bovine plasma albumin. The inhibition of β-glucuronidase by hyaluronic acid and heparin (Becker & Friedenwald, 1949), chondroitin sulphate (Walker & Levvy, 1953) and DNA (Smith & Mills, 1953) has been noted previously.

It is suggested that similar inhibition of acid hydrolases may occur in many tissue extracts. Estimations of the 'free activity' of the enzyme may be influenced by the presence of negatively charged molecules (e.g. the acid mucopolysaccharides investigated in this work), and by the

quantity of protein and of cations available to compete with the enzyme for the polyanion. To facilitate comparison of the activities of acid hydrolases in tissue extracts, or of enzyme fractions during purification, it may be desirable to add an excess of enzymically inactive protein.

I wish to thank Miss K. Broady for skilled technical assistance.

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Isolation of Rat-Skin Lysosomes and a Comparison with Liver and Spleen Lysosomes

By C. H. Wynn and M. Iqbal. (*Department of Biological Chemistry, University of Manchester, Manchester*, 13)

Skin, being tough and elastic, is unsuitable for homogenization without pretreatment. Attempts to prepare homogenates after treatment with trypsin, ficin, pancreatin or collagenase were unsuccessful. The epidermis of dorsal skin, which had been plucked four days previously, could be separated by the stretch method of Van Scott (1952) but subcellular fractionation of an homogenate of this epidermis yielded insufficient lysosomes for further detailed study. Finally, the dorsal skin of 5–8-day old rats was removed and, after removing the subcutaneous fat, an homogenate was easily prepared. Subcellular fractionation by a slightly modified method of De Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955) gave reasonable yields of lysosomes. Liver and spleen lysosomes were prepared similarly.

Both lysosomes and homogenates were prepared in 0·25M-sucrose adjusted to pH 5·2 with 0·05M-tris–maleate and incubated at 0° and 37°. At various time intervals, free acid phosphatase activity was determined using a 10min. assay under isotonic conditions and the results expressed as a percentage of the total activity.

The free activity of liver homogenates rose from 20% at zero time to 95% after incubation at 37° for 3hr., while at 0° there was no liberation of activity on incubation. Similarly, the free activity of liver lysosomes was 5% at zero time, 80% at 1hr., and rose to 95% after 3hr. at 37°. A negligible increase in free activity of liver lysosomes was observed at 0°.

The free activity of spleen homogenates increased from 14% at zero time to 45% after 3hr. at 37°. Spleen lysosomes showed little increase after 30min. at 37° but from the initial 20%, 59% of the activity was free after 3hr. These results are similar to those obtained by Rahman (1964).

The initial level of free activity of skin homogenates was very high (55%) but on subsequent incubation at 37° for 3hr. it increased only to 65%. Even more striking was the finding that the free activity of skin lysosomes only increased from 6% to 24% after incubation for 3hr. at 37°. At 0° both homogenate and lysosomes remained unaffected.

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The Nature of the Collagenolytic Activity of Rat-Liver Lysosomes

By C. H. Wynn and M. A. Wahid. (*Department of Biological Chemistry, University of Manchester, Manchester*, 13)

It has been reported (Frankland & Wynn, 1962) that partially purified preparations of rat-liver lysosomes degrade acid-soluble collagen to a limited extent. Further studies revealed that, during the course of this degradation, there was very little peptide bond breakage but significant release of free carboxyl groups did occur. This suggested that a possible site of action of the lysosomal preparation was the ester bonds present in the collagen macromolecule (Wynn, 1963).

In the present study, lysosomes were solubilized by treatment with deoxycholate, the soluble protein separated from deoxycholate by gel filtration on Sephadex G-25, and fractionated on DEAE-cellulose using a concave gradient of 0·01M-tris–acetic acid, pH 8·5 containing 0·1 to 0·0M-sodium acetate. This procedure revealed the presence of at least five distinct esterase activities in the lysosomal preparation but only one fraction capable of degrading collagen. This fraction was coincident with one of the main esterase peaks and attempts at further separation were unsuccessful. This fraction was homogeneous on paper electrophoretic and ultracentrifugal analysis.

The response of the two activities to inhibitors was essentially the same. Both were strongly inhibited by fluoride, weakly inhibited competitively by physostigmine, and unaffected by *p*-chloromercuribenzoate. These results are suggestive of the identity of the two activities.

The hydrazinolysis of acid-soluble collagen which

had been previously treated with this enzyme revealed that no rupture of ester-like bonds had occurred under the experimental conditions (Gallop, Seifter & Meilman, 1959). Ultracentrifugal analysis of the collagen after treatment also failed to show any significant changes in the size of the molecule. Preliminary evidence suggests that during the enzyme action some *N*-terminal tyrosine is released and thus the action of the enzyme on undenatured collagen may be confined to the teleopeptide portion of the molecule.

On the other hand, heat-denatured collagen was readily split into dialysable peptides, rich in hydroxyproline, suggesting attack on the previously crystalline regions of the collagen. Ultracentrifugal analysis showed that the collagen had been extensively degraded. This difference in action on native and denatured collagen suggests that the tertiary structure of collagen offers steric hindrance to the enzyme.

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Inhibition of Connective Tissue Proteases by Antimalarial, Antirheumatic Drugs

By M. W. Whitehouse and F. K. Cowey. (*Department of Biochemistry, University of Oxford*)

After incubating fresh beef nasal- or tracheal-cartilage slices at pH 5·0 and 37°, hydroxyproline-containing fractions and a chondromucopeptide (CMPep) were recovered from the medium. The greater part of this material (80%) arose through the activity of endogenous (autolytic) enzymes in the cartilage, indicated by (*a*) the low yields on incubating cartilage pre-dried at 120° and (*b*) the fact that a bacteriostat (0·25mM-thiomersal) had little effect on the amount of tissue-degradation products released. The yield of CMPep, determined colorimetrically with orcinol (Moretti & Whitehouse, 1963) or turbidometrically with Rivanol (Whitehouse & Boström, 1961), was optimal at pH 5·0 (16 to 20% total tissue glucuronosidate solubilized in 20hr.). The presumed 'collagenase' activity was greater at pH 5 (1μg. hydroxyproline/mg. cartilage dry wt./20hr.) than at pH 7.

High levels (20mM) of mepacrine, chloroquine, hydroxychloroquine and primaquine salts in the incubation medium inhibited these autolytic reactions by more than 50%. These drugs did not interfere with methods for determining CMPep. The potency of these drugs was much greater if they were pre-incubated at pH 7·4 (up to 6hr.) with cartilage slices before determining CMPep release at pH 5·0 (into drug-free medium).

The chloroquine-sensitivity of the chondromucoprotease was unaltered by pretreating cartilage with detergents, e.g. Triton X-100, or by repeated freezing and thawing, suggesting that the drug was not merely stabilizing lysosomes to prevent liberation of an intracellular protease (cf. Weissmann, 1964). Furthermore, a chloroquine-sensitive enzyme could be extracted in poor yields from homogenized cartilage, which degraded chondromucoprotein preparations (Malawista & Schubert, 1958).

Collagenases from rat skin (Goldstein, Patel & Houck, 1964) and *Clostridium histolyticum* (Sigma Chemical Co. London) were also inhibited by 10mM-chloroquine. Chloroquine inhibition of gelatine hydrolysis at pH 7 by this bacterial collagenase was non-competitive with respect to substrate. Chloroquine (25mM) did not affect the hydrolysis of either (i) gelatine, casein and albumen, by papain and trypsin or (ii) cartilage, chondroitin sulphate and hyaluronate by ovine testicular hyaluronidase.

Both chloroquine and hydroxychloroquine were degraded by cartilage slices yielding desethylchloroquine as the principal metabolite. This compound was equipotent with chloroquine as an inhibitor of chondromucoprotease and collagenases. 4-Amino-7-chloroquinoline was much less potent than chloroquine, whilst side-chain fragments (potential metabolites) of the chloroquine molecule (e.g. 2-amino-5-diethylaminopentane, 5-diethylaminopent-2-one) were inactive.

These findings indicate that basic antirheumatic drugs may, like acidic anti-inflammatory drugs (Whitehouse & Skidmore, 1965), specifically inhibit certain proteases and protect connective tissues in inflammatory conditions.

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Glycopeptides in Human Urine

By E. H. F. McGale and F. R. Jevons. (*Department of Biological Chemistry, The University, Manchester*, 13)

The presence of glycopeptides in human urine has been established by a number of workers (e.g.

Bourrillon, Cornillot & Got, 1962; Hakomori & Ishimoda, 1962; Lundblad, 1965).

In the present study, interference from the urinary pigments in the detection and estimation of carbohydrate was observed, in particular with reagents containing strong sulphuric acid, such as in the orcinol method for neutral sugars (Hartley & Jevons, 1962). A differential method was therefore used, based on the production of a colour from the pigments on heating only with sulphuric acid of corresponding strength. The absorption spectrum between 360mμ and 640mμ of such mixtures was quite distinct from that formed when orcinol was present too, but the difference spectrum corresponded closely to that given by sugars with the orcinol-sulphuric acid reagent. The difference in absorption at 500mμ was therefore used in the analysis of chromatographic fractions for neutral sugars. Similar corrections were applied in analyses for methylpentose by the cysteine–sulphuric acid method (CyR 10 of Dische & Shettles, 1948) and uronic acid by the carbazole–sulphuric acid method (Bitter & Muir, 1962).

A glycopeptide-containing fraction was obtained from pooled male urine by adsorption on a sulphonic acid resin (Zeo-Karb 225, H^+-form) and elution with 5N-ammonia. The resultant fraction (ammonia eluate, 1·0–1·5g./l. of urine) contained hexose (0·9%), methylpentose (0·9%), hexosamine (0·4%), sialic acid (0·04%) and uronic acid (0·08%). The fraction of ammonia eluate precipitated by ethanol between 50% and 90% concentration (v/v) was chromatographed on a column of Zeo-Karb 225, NH_4^+-form, using stepwise elution with ammonium formate and ammonium acetate buffers, over a pH range 3·08–6·80. The first fractions eluted showed 3 major peaks of material containing combined neutral sugar almost free of pigment. The 4 major sugar-containing peaks later eluted were each coincident with pigment peaks. In each of these 4 peaks, glucose, galactose, mannose and fucose were detected by hydrolysis and paper chromatography.

These observations could indicate that some of the urinary carbohydrate is linked to pigment.

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Degradation of Mucoprotein Carbohydrate by Ox-Liver Preparations

By T. J. Langley and F. R. Jevons. (*Department of Biological Chemistry, The University, Manchester*, 13)

Previous work has shown that *N*-acetylglucosamine can be removed from ovomucoid preparations by an enzyme system from ram testis (Caygill & Jevons, 1964). Acetic acid extracts were fractionated by ammonium sulphate precipitation and chromatography on CM-cellulose, and the complexity of the system was shown by the synergistic effects observed on combining various peaks (Roston, Caygill & Jevons, 1965).

Similar enzyme systems have now been identified in rat and ox livers. The fraction of an aqueous ox-liver homogenate precipitating between 20 and 60% saturation of ammonium sulphate (P_{60}) has been shown to be capable of effecting extensive degradation of the carbohydrate of ovomucoid; as estimated by a slight modification of the method of Reissig, Strominger & Leloir (1959), up to 90% of the *N*-acetylglucosamine present could be released on prolonged incubation.

The presence of β-acetylglucosaminidase (Weissmann, Hadjiioannou & Tornheim, 1964) and β-galactosidase (Levvy, McAllan & Hay, 1962) in the ox-liver homogenate and P_{60}, was shown using *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide and *o*-nitrophenyl-β-D-galactoside respectively as substrates. In addition, an α-mannosidase has been demonstrated with *p*-nitrophenyl-α-D-mannoside; it shows a pH optimum in 0·05M-sodium citrate buffers at pH 4·3. More than 70% of all three activities were recovered in P_{60}. By stepwise elution between pH 4·0 and 6·0 from a column of CM-cellulose, six fractions were obtained, of which the second (CM_{II}) contained most of all the three glycosidases. When CM_{II} was put on a column of Sephadex G-75, maxima of β-acetylglucosaminidase and α-mannosidase activity were in tube 10 and that of β-galactosidase in tube 13.

CM_{II} and tube 10 from the Sephadex column were 3 and 8 times more powerful respectively in effecting initial release of *N*-acetylglucosamine from ovomucoid, which runs roughly parallel with β-acetylglucosaminidase activity. However, the total amount of *N*-acetylglucosamine released was lower with the more purified fractions. Even on prolonged incubations with further additions of enzyme, only 68% and 50% respectively of the *N*-acetylglucosamine present was released. This indicates that enzymes other than the three specifically identified so far must be involved in effecting extensive degradation.

N-Acetylglucosamine release from ovomucoid by P_{60} was inhibited by added *N*-acetylglucosamine;

since such inhibition has been reported for β-acetylglucosaminidase in beef liver and rat kidney (Watanabe, 1936; Pugh, Leaback & Walker, 1957), this is consistent with the participation of this enzyme in the degradation system.

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Antiserum to α-Elastin and its Use in the Detection of Soluble Elastin in Extracts of Foetal Ligaments

By D. S. Jackson, L. S. Sandberg and E. G. Cleary. (*Department of Medical Biochemistry, The University, Manchester*, 13)

α-Elastin was prepared from purified bovine ligamentum nuchae by a modification of the method described previously (Partridge, Davis & Adair, 1955).

A 2% solution of α-elastin, mixed and emulsified with equal parts of Freund's adjuvant, was injected into rabbits at weekly intervals for seven weeks. After this time a weak precipitin was detected. After two months rest a series of four secondary injections was given at weekly intervals and the series of secondary injections repeated twice with a two-month rest between each series.

A stronger precipitin reaction was then obtained. The Ouchterlony diffusion technique revealed the presence of one precipitin band, but more clear-cut results were obtained by immunoelectrophoresis. All control sera were negative and no cross-reaction was observed with bovine sera or collagen. Antigen-antibody interaction was also demonstrated by cutaneous anaphylaxi using guinea-pigs.

Haemagglutination studies were also carried out using formalized, tanned red cells (Butler, 1963). A strong positive reaction was obtained and a cross-reaction demonstrated with soluble K-elastin (Robert, Parlebas, Poullain & Robert, 1963) and with α-elastin from foetal tissue.

Extracts of foetal bovine ligamentum nuchae were prepared as previously described (Jackson, Sandberg & Cleary, 1965). Haemagglutination was carried out using the ligament extracts as antigen and anti α-elastin serum as antibody. A strong cross-reaction was observed. The ligament extracts were also used to immunize rabbits using the same technique as that described for α-elastin. The resulting anti-serum was found to react with α-elastin using the haemagglutination technique.

Fractionation of the ligament extract has been attempted using Sephadex G-200 and DEAE-cellulose and the fractions obtained monitored by the haemagglutination technique for the presence of soluble elastin. A positive reaction has been found with one particular fraction only. However, amino acid analysis indicates it is still contaminated with a non-elastic protein with a high content of polar amino acids. Efforts at further purification have so far been unsuccessful, but our results so far would suggest the presence in extracts of foetal ligament of a soluble elastin.

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The Inhibition of Mucoprotein Biosynthesis in the Sheep Colonic Mucosa, *in vitro*, by Salicylic Acid

By A. Allen and P. W. Kent. (*Department of Biochemistry, University of Oxford*)

Considerable evidence suggests that the formation of mucosubstances in cells is sensitive to the presence of salicylates. In cartilage slices, for example, the incorporation of $^{35}SO_4^{2-}$ as well as the Q_{O_2} has been found to be diminished by such inhibitors (reviewed by Dixon, Martin, Smith & Wood, 1963, and Whitehouse, 1965).

The present investigations have been concerned with the influence of salicylate upon the incorporation of isotope from [2-^{14}C]glucose into mucoprotein by surviving sheep colonic mucosae (Draper & Kent, 1963). Washed mucosal scrapings were incubated in Krebs–Ringer III medium (in which glucose had been replaced by tracer amounts of [2-^{14}C]glucose and L-glutamine (3·2mM final concentration) had been added) at pH 7·4 and at 37° for up to 3hr. When salicylate was present in a final concentration of 3·75mM the Q_{O_2} was −2·2, compared with −2·9 in control experiments (after 3hr.). At higher concentrations of the inhibitor, up to 30mM, the Q_{O_2} decreased progressively to −0·30.

In parallel experiments, the incorporation of isotope from [2-^{14}C]glucose (10 to 12·5μc in each experiment) also decreased with increasing amounts of inhibitor. Thus, after 2·5hr. in the presence of 3·75mM-salicylate, 19·3% incorporation of the added ^{14}C was observed (average of three experiments). In control experiments, the radioactivity of the ^{14}C-mucoprotein was 13500 counts/min./mg. (average of three experiments) i.e. 30·4% of the added isotope. In the presence of 15mM and 30mM-salicylate, 1·3 and 0·03% radioactive incorporations were obtained.

In the ^{14}C-mucoprotein obtained in control experiments, i.e. salicylate-free, the radioactivity was distributed amongst all the monosaccharide constituents (measured on the sugars, separated chromatographically after acidic hydrolysis). In a partly inhibited system, in the presence of 3·75mM-salicylate the total labelling the mucoprotein was decreased by 41% and each monosaccharide constituent by the amount stated (relative to control): *N*-acetylneuraminic acid (73%), *N*-glycollylneuraminic acid (41%), D-glucosamine (53%), D-galactosamine (39%), L-fucose (13%), D-galactose (6%).

It appears therefore that the inhibition of mucoprotein biosynthesis is due not only to a general curtailment of the energy supply, but also to a selective action on the formation of the amino-sugar constituents. This may be consistent with the known inhibitory effect of salicylate on fructose 6-phosphate transaminase (EC. 2.6.1.16, Bollet, 1961).

The results indicate the ready suppression of mucoprotein biosynthesis in a tissue of the gastrointestinal tract, which if applicable to other such tissues, may be of general interest in relation to the side-effects of salicylates when administered therapeutically.

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Species Differences in the Metabolism of some Methoxy-6-Sulphanilamidopyrimidines

By J. W. Bridges, M. R. Kibby and R. T. Williams. (*Department of Biochemistry, St Mary's Hospital Medical School, London, W. 2*)

A number of the commonly used 'long acting' sulphonamide drugs contain a methoxy-substituted pyrimidine ring. In order to determine the relationship between their structure, metabolism and retention in the body we have investigated the fates of 2-, 4- (sulphamonomethoxine), 5-, 2,4- (sulphadimethoxine, Madribon), 2,5- and 4,5- (sulphorthodimethoxine, Fanasil) methoxy-6-sulphanilamidopyrimidines in the rat, rabbit, monkey and man.

In a previous communication (Bridges, Kibby & Williams, 1965) we have shown that the major metabolite of sulphadimethoxine in man is the N^1-glucuronide (S–N^1–G). This metabolite accounted for 62% of the dose recovered in the 24hr. urine, while the N^4-acetylsulphadimethoxine (N^4–Ac–S) accounted for a further 22%. We have now shown that the rhesus monkey metabolizes sulphadimethoxine similarly to man (S–N^1–G, 62–70%, N^4–Ac–S, 8–21%). In the rat and the rabbit however the major metabolite was N^4–Ac–S, and only small amounts of S–N^1–G (7%) were formed by the rat and none by the rabbit. Sulphadimethoxine thus shows a marked species difference in its metabolism.

No other methoxy-6-sulphanilamidopyrimidine studied was metabolized to its corresponding N^1-glucuronide to any significant extent in any of the species examined. The major metabolite of each sulphonamide was the corresponding N^4-acetyl derivative except in the case of the 2,5-dimethoxy compound (0–7% N^4–Ac) which was excreted almost entirely as the free drug. N^4-Glucuronides and N^4-sulphates were formed as minor metabolites (less than 11%) of all the sulphonamides.

Since sulphadimethoxine alone of the compounds studied is converted in significant amounts to an N^1-glucuronide the structural requirements for the formation of this conjugate would appear to be quite specific. In preliminary experiments we have found that the formation of S–N^1–G can be brought about by rat-liver slices although the extent of conjugation was small.

4,5-Dimethoxy-6-sulphanilamidopyrimidine showed an outstanding persistence in man, but not in other mammals and was excreted at a rate of about 5% per day compared with rates of 20–30% for the other sulphonamides studied.

One of us, M. R. K., is grateful to May and Baker Ltd., for a grant.

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Dealkylation of Diethyltin by the Rat

By J. W. Bridges, D. S. Davies and R. T. Williams. (*Department of Biochemistry, St Mary's Hospital Medical School, London, W. 2*)

Although organo-tin compounds are extensively used as fungicides, bactericides, anthelmintics and

as plastics stabilizers, relatively little is known about their metabolism (Barnes & Stoner, 1959).

We have studied the metabolism of ethyltin trichloride, diethyltin dichloride and diethyltin oxide in the rat. On oral administration of ethyltin trichloride (25mg./kg.) over 90% of the tin was excreted in the faeces and less than 2% in the urine. Ethyltin trichloride given intraperitoneally (12·5 mg./kg.) was largely excreted unchanged in the urine, trace amounts only were found in the bile. Thus it appears that ethyltin like inorganic tin is poorly absorbed from the gut.

Rats given di[1-^{14}C]ethyltin dichloride (10mg./kg.) intraperitoneally excreted about 95% of the Sn and 55% of the ^{14}C in the urine or faeces in 4 days. A third of the Sn was excreted in the urine mostly as ethyltin (80–85%) together with small amounts of diethyltin (10–15%). The Sn in the faeces consisted of ethyltin (50%) diethyltin (16%), the remainder probably being inorganic tin. Injected diethyltin dichloride was excreted in the bile almost entirely as diethyltin (66% of the dose). Di[1-^{14}C]ethyltin oxide gave similar results when given orally. Thus diethyltin is considerably dealkylated in the rat and our evidence suggests that this reaction may occur in both the gut and the tissues.

Despite this extensive dealkylation no $^{14}CO_2$ was detected in the expired air implying that the ethyl group was not oxidatively attacked. If such an attack occurred at a Sn–C bond it would be expected to be preferentially directed towards the Sn atom in which case the ethyl group could be removed as ethane. In our experiments about 40% of the ^{14}C was unaccounted for and we conclude that it was lost as [1-^{14}C]ethane which would not have been trapped under our experimental conditions.

The dealkylation of diethyltin is a true detoxication reaction since diethyltin dichloride is approximately four times more toxic than monoethyltin trichloride.

One of us, D. S. D., is grateful to Dr Salsbury's Laboratories for a grant.

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The Isolation of Progesterone from Human Pregnancy Urine

By A. A. A. Ismail and R. A. Harkness. (*Medical Research Council Clinical Endocrinology Research Unit, University of Edinburgh*)

A number of unsuccessful attempts have previously been made to isolate progesterone from human urine (Marker, Kamm & McGrew, 1937; Drosdowsky *et al.* 1965). However, two studies with radioactive progesterone have shown that about 0·01% of an intravenous dose of this hormone is probably excreted unchanged in the urine (see Drosdowsky *et al.* 1965). The further development of a modification of the Girard reaction originally described by Teitelbaum (1958) has made it possible to separate quantitatively a 'conjugated ketone' fraction containing progesterone from crude urinary extracts (Ismail & Harkness, to be published).

Progesterone was isolated from about 50l. of urine from a woman in late pregnancy. 10l. batches of urine, after storage for no longer than 1 week, were extracted with 2vol. of ether. The extracts were dried with anhydrous sodium sulphate and evaporated to dryness. The residue was subjected to the modified Girard separation and the 'conjugated ketone' fraction was chromatographed on paper in the system petroleum–methanol–water (100, 80, 20 by vol.). The area corresponding to progesterone was eluted and chromatographed on thin layers of silica gel G in the system hexane–ethyl acetate (5:1·5, v/v); the progesterone area was eluted. Portions of this eluate were subjected to gas–liquid chromatography on Gas-chrom P coated with 1% (w/w) SE-30 or QF-1. A stream-splitter (4:1) was used and three components were isolated from the larger stream, one of which had the same retention time as progesterone. The infrared absorption spectrum of this component determined by the micro-method of Sykes & Kelly (personal communication) was identical with that of authentic progesterone between 2 and 15μ. The ultraviolet absorption spectrum between 220 and 400mμ also corresponded to that of progesterone. On oxidation with chromium trioxide and reduction with potassium borohydride this compound behaved like progesterone. It was estimated from gas chromatographic peak heights that there was no more than 250μg. of progesterone in the pooled urine residues.

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Metabolism of [4-^{14}C]Testosterone by Human and Canine Prostate

By M. A. Aliapoulios, J. Chamberlain, Nada Jagarinec and P. Ofner. (*Lemuel Shattuck Hospital and Harvard School of Dental Medicine, Boston, Massachusetts, U.S.A.*)

It is not known whether testosterone itself or biologically active, prostatic metabolites exert the

well-known trophic stimulation of the gland (Hilgar & Hummel, 1964). Both the aging human and canine prostates can develop benign and malignant growths. We have therefore selected the dog for studies of prostatic uptake and metabolism of the male hormone.

Human prostate with benign hypertrophy (bph) was obtained at surgery and canine bhp tissue from an animal killed by an overdose of Nembutal. Homogenates and subcellular fractions were prepared as described by Chamberlain, Jagarinec & Ofner (1965). In preliminary experiments with homogenates of human bph tissue, we established incubation conditions (3·5 μg. substrate/g. tissue dry wt.) for almost complete conversion of substrate to at least 10 transformation products. The human tissue homogenate contained 3α-, 3β- and 17β-hydroxy steroid dehydrogenases and Δ^4-3-oxo steroid 5α- and 5β- reductases. Most of the 17β-hydroxy steroid dehydrogenase activity was in the mitochondria, which showed little other activity. The 3α- and 3β-hydroxy steroid dehydrogenases and the 5β-reductases were located in the high-speed supernate (hss). The 5α-reductase was located in both microsomal and hss fractions. Only 17β-hydroxy steroid dehydrogenase and 5α-reductase were found in canine bph homogenate; considerable 17β-hydroxy steroid dehydrogenase activity was detected in all subcellular fractions and 5α-reductase in none.

In dog experiments *in vivo*, 2 young animals were sacrificed (I) 30 min. and (II) 60 min. after direct infusion of [4-^{14}C]testosterone into the prostate and the gland and plasma investigated for radiometabolites. Uptake of steroid was 0·1 μg./g. prostate tissue (dry wt.) for Dog I and 0·02 μg./g. for Dog II. In both experiments 17-oxo steroids and 5α-reduced steroids were found in prostate and plasma; a significant level of diols could be demonstrated only in the prostate tissue.

The results of our experiments encourage us to believe that the circulating Δ^4-3-oxo steroids are completely metabolized after uptake by prostate gland.

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **455th Meeting** *of the Society was held at Guy's Hospital Medical School, London, S.E.*1, *on Friday,* 17 *December* 1965, *when the following papers were presented*

COLLOQUIUM ON 'SOME ASPECTS OF LIPID BIOCHEMISTRY OF THE NERVOUS SYSTEM'

Lipid Metabolism in Relation to Brain Structure

By A. N. Davison. (*Biochemistry Department, Guy's Hospital Medical School, London, S.E.* 1)

It is now recognized that almost all the lipids of the central nervous system are associated with membranes (Seminario, Hren & Gomez, 1964). Although much of this lipid forms an essential part of the membrane structure, lipid synthesis and direct participation of lipid in enzyme reactions may also be associated with membrane systems (Green & Fleischer, 1964). Since it seems likely that structural lipid is relatively more stable than non-structural an assessment of the part played by lipid in neural membrane structure may be obtained from long-term metabolic studies using radioactive isotopes. Thus a major part of the myelin sheath lipid has been found to undergo very slow turnover at the same rate as the myelin protein (Cuzner, Davison & Gregson, 1965). Short-term experiments using isotopes suggest that there is a rapidly metabolized component in myelin (Eichberg & Dawson, 1965) but this probably represents only a very small fraction of the whole. These metabolic results for myelin constituents harmonize with the proposition that myelin is composed of membrane subunits of lipid and protein (Gent, Gregson, Gammack & Raper, 1964). The composition and metabolism of other brain subcellular structures differs from that of myelin (Eichberg, Whittaker & Dawson, 1965; Khan & Wilson, 1965; Davison & Gregson, unpublished results). In the case of rat-brain mitochondria much of the lipid appears to be a representative part of the organelle structure, because the composition and turnover characteristics of a deoxycholate membrane fraction and that of the whole organelle are similar. Rats of different ages have been injected with different isotopes (Khan & Wilson, 1965; Cuzner, Davison & Gregson, unpublished results) and the metabolism of mitochondrial lipid determined after long periods. The whole organelle lipid undergoes turnover with a half-life of approximately 30 days; that is three times slower than the turnover found for liver mitochondria (Fletcher & Sanadi, 1961). Individual labelled lipids were separated by thin-layer chromatography. There seemed to be little loss of incorporated radioactivity from cholesterol, cerebrosides or sphingomyelin in these mitochondrial preparations although other labelled phosphatides present underwent turnover. Some attempt will be made to interpret these results in comparison with the metabolic behaviour of the myelin sheath lipids.

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Some Structural Aspects of Lipids in Membrane Function

By L. L. M. Van Deenen, L. M. G. Van Golde and R. A. Demel. (*Department of Biochemistry, University of Utrecht, The Netherlands*)

The molecular species of natural phosphoglycerides were recognized by separation of intact phospholipids or enzymically produced diglycerides on silicic acid impregnated with silver nitrate, combined with a determination of the fatty acid localization through the use of snake-venom phospholipase A and pancreatic lipase respectively. Depending on the origin of the phospholipid up to 23 species were detected and significant quantitative differences were found to exist in the proportions of various species, e.g. between lecithin from liver, erythrocytes and brain. A comparison with the force–area characteristics of monomolecular layers of defined synthetic lecithin species explained the

differences in molecular packing between monolayers of different natural phosphoglyceride preparations, the films of the latter preparations, however, being all of the liquid-expanded type.

Dietary experiments demonstrated that several membranes of animal tissues are able to preserve the physicochemical properties of a given phosphoglyceride class within certain limits by (*a*) a positionally specific interchangeability of fatty acid constituents of related polarity; and (*b*) changes in the proportions of the individual molecular species of the phospholipid concerned.

The orientation of lipid molecules in monomolecular films depends not only on the nature of the paraffinic chains of the phospholipids but also on the phospholipid/sterol ratio. The reduction of the molecular area of individual phospholipid species by cholesterol was investigated with films containing synthetic phospholipids. The magnitude of this effect appeared to depend largely on the nature of the fatty acid constituents of the phospholipid. For example, the mean area per molecule in mixed films of cholesterol with (distearoyl)-3-lecithin and (didecanoyl)-3-lecithin did practically follow the simple additivity rule, whereas the condensing effect of cholesterol was evident with films of (1-stearoyl-2-lauroyl)-3-lecithin, (dimyristoyl)-lecithin, (1-stearoyl-2-oleoyl)-3-lecithin as well as with (dioleoyl)-3-lecithin. However, in the expanded films of synthetic phosphoglycerides containing linoleic or linolenic acid no appreciable reduction of the phospholipid cross-section was induced by cholesterol. Although it is not permitted to extrapolate these results to complex biological structures a comparison of these observations with fresh data on the make-up of the molecular species of phospholipids in membranes endorses the view that particularly in the myelin sheath cholesterol may contribute to attain a highly organized alignment of the lipid constituents. Long-chain fatty acid esters of cholesterol are either absent (e.g. myelin) or present in small concentrations only (erythrocytes) in a number of membranes. The interfacial behaviour of these compounds indicates that there is a difference in suitability between cholesterol and cholesterol esters to serve as membrane constituents. The abundance of cholesterol esters in a number of demyelinating diseases may contribute to the disorganization of the membranes concerned.

Lipids and Membrane Phenomena in Neural Systems

By D. B. Gammack. (*Department of Biochemistry, Institute of Psychiatry, Maudsley Hospital, Denmark Hill, London, S.E.* 5)

The integrity of the lipid layers of the boundary membranes of nerve cells is essential for the maintenance of the differential concentrations of Na^+ and K^+ on which normal resting and action potentials depend. Thus phospholipases injected into the axoplasm of the squid giant axon render the axon inexcitable (Tobias, 1960). Furthermore it has been shown that the excitability of cerebral-cortical tissues which is lost on their exposure to polybasic substances can be restored by the application of polyacids, acidic lipids being particularly effective (McIlwain, 1964). This suggests that the selective movement of cations across the cell membranes depends on the availability of anionic groups with an adequate electrostatic field strength such as phosphate and carboxylate groups on lipids and proteins which may function as ion-specific sites in the membrane (Eisenman, 1961).

Recent studies have shown that acidic lipids including cerebral phospholipids and glycolipids show defined ion-exchange properties. Differential binding of Na^+ and K^+ by phosphatidylserine, phosphatidic acid and sulphatides has been demonstrated on titration of aqueous micellar dispersions of the lipids (Abramson, Katzman & Gregor, 1964, 1966; Abramson, Katzman, Wilson & Gregor, 1964). Selectivity between Na^+ and K^+ has not generally been observed when lipids are orientated in monolayers (Rojas & Tobias, 1965; Gammack & Raper, 1966) and in bilayers (Mueller, Rudin, Tien & Wescott, 1964).

Cation selectivity coefficients observed for isolated lipids are smaller in magnitude than those shown for synthetic ion-exchangers (Bregman, 1953) and for the squid giant axon for which differential permeability to Na^+ as against K^+ of the order of 10 or more has been reported. Ion selectivity in such neural membranes may thus depend on the specific orientation and spacing of the polar groups of lipid molecules as might occur in the lining of membrane pores or, in the light of the greater selectivity of lipids when in micellar form, it may depend on a micellar organization of the lipids in the membranes (Lucy, 1964).

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Phosphoinositides of Nervous Tissue

By R. M. C. Dawson. (*Biochemistry Department, A.R.C. Institute of Animal Physiology, Babraham, Cambridge*)

The nervous system contains three phosphoinositides, monophosphoinositide, diphosphoinositide and triphosphoinositide, the latter two in much higher concentrations than in other tissues. It also contains enzymic systems catalysing both the stepwise phosphorylation of monophosphoinositide through diphosphoinositide to triphosphoinositide and the reverse stepwise dephosphorylation. These systems appear to be active in the intact brain since the phosphomonoester groups of polyphosphoinositides become rapidly labelled with ^{32}P after the injection of labelled phosphate. Moreover some of the polyphosphoinositides disappear rapidly from brain and peripheral nerve within a few minutes after death.

Evidence is accumulating that the polyphosphoinositides, and in particular triphosphoinositide, are selectively localized in the myelin sheath. When brain tissue is fractionated the polyphosphoinositides are found to be associated mainly with the myelin-rich fractions and when very pure myelin is prepared the concentration of polyphosphoinositide in its phospholipids is some 2 to 3 times greater than in the whole-brain homogenate. Polyphosphoinositides appear in the nervous system during the period of maximum myelination and, moreover, the concentration differences between white and grey matter and between myelinated and non-myelinated nerve fibres support the theory that they are largely localized in the myelin sheath.

In adult animals phosphomonoester groups of the myelin polyphosphoinositides exchange very rapidly with injected [^{32}P]phosphate, in contrast to the other phospholipids which are relatively inert. Myelin-rich subcellular particles from brain synthesize ^{32}P-labelled triphosphoinositide when incubated with [γ-^{32}P]ATP.

In myelin there is evidence that the polyphosphoinositides are present as Ca–Mg salts of complexes with proteins which are not necessarily the same as the phosphatido-peptide complexes which have been isolated from chloroform–methanol extracts of brain tissue. So far there is no firm evidence as to the physiological role of polyphosphoinositides in nervous tissue although one suggestion is that their affinity for bivalent ions, especially Ca^{2+}, may play a part in controlling the permeability of certain areas of the nerve membrane to univalent ions.

Phospholipid Breakdown in the Central Nervous System

By G. R. Webster. (*Department of Chemical Pathology, Guy's Hospital Medical School, London, S.E.* 1)

Although loss of phospholipid from degenerating nervous tissue and from normal nervous tissue incubated *in vitro* has been known for a number of years, little specific information has, until recently, been available about the enzymic reactions concerned in this process in the nervous system.

Gallai-Hatchard, Magee, Thompson & Webster (1962) have demonstrated phospholipase A-like activity (phosphatide acylhydrolase) in brain releasing a free fatty acid and lysolecithin from lecithin emulsions. Phosphatidylethanolamine and phosphatidylserine were also shown to be split to give the corresponding lyso derivatives. More recently Ansell & Spanner (1965) have described an enzyme in rat brain cleaving the vinyl ether linkage of ethanolamine plasmalogen and releasing an equivalent amount of alkali-labile phospholipid. Earlier work (Dawson, 1956; Marples & Thompson, 1960) had shown that brain contains a phospholipase B (lysolecithin acylhydrolase). An active phosphodiesterase splitting glycerophosphorylcholine to free choline and glycerophosphate is also present in nervous tissues (Webster, Marples & Thompson, 1957). These studies have thus demonstrated that mammalian brain possesses the necessary enzymes for the complete degradation of glycerophosphatides, such as lecithin, to fatty acids and water-soluble derivatives.

Because of the cytolytic effects of lysophosphatides particular interest has centred round the metabolism of these substances. Lands (1960) described the enzymic acylation of lysolecithin, with acyl-CoA as intermediate, to form lecithin in rat liver. This reaction has now also been shown in many other tissues including those of the nervous system (Webster & Alpern, 1963; Webster, 1965). Activity was demonstrable in homogenates of rat brain and is

present in both mitochondrial and microsomal fractions. Lands and his co-workers suggested that phospholipases and the acylation system might form a cyclical mechanism mediating independent turnover of fatty acids in glycerophosphatides. In an attempt to obtain evidence in brain for this hypothesis, slices of rat brain were incubated in a physiological medium in the presence of either O_2 or N_2. Subsequent analysis of the lipids showed accumulation of lysolecithin which was much more marked under anaerobic conditions (Webster & Thompson, 1965). These experiments thus provide further evidence for the presence of phospholipase A activity in fresh brain preparations and are consistent with the view that the resulting lysophosphatides can be re-acylated. Similar experiments in which ^{14}C-labelled long-chain fatty acids were added to the incubation medium have shown incorporation of these into lecithin and to a lesser extent into phosphatidylethanolamine.

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The Patterns of Gangliosides in Mental and Neurological Disorders

By L. Svennerholm. (*Department of Neurochemistry, Psychiatric Clinic II, University of Gothenburg, Sweden*)

The gangliosides are predominantly confined to neural tissues in which they occur in the neuronal cytoplasm, dendrites and axons. There are four major gangliosides (G_1) which all have the same neutral carbohydrate moiety, galactosyl-(1→3)-*N*-acetylgalactosaminoyl-(1→4) galactosyl-(1→4)-glucosyl, but varying number of the sialic acids which are bound to C-3 of the two galactose moieties (reviewed by Svennerholm, 1964). Glucose is attached to the primary hydroxyl group of sphingosine by a glycosidic bond. C_{18}-Sphingosine is predominant in foetal gangliosides but, with increasing age, C_{20}-sphingosine increases and constitutes about 60–70% of total sphingosines in senile brain gangliosides. In contrast with other glycolipids which contain mainly very long-chain fatty acids (C_{22}–C_{26}), and a high proportion of hydroxy fatty acids (brain galactolipids and kidney glycolipids), brain gangliosides contain 80–90% of stearic acid (18:0).

Other than the major gangliosides, normal brains contain less than 10% of four other brain gangliosides, mono- and di-sialogangliotrihexosides (G_2), lacking the terminal galactose, and mono- and di-sialogangliolactosides (G_3). Recent data suggest that these gangliosides can be either precursors or degradation products of the major gangliosides.

Klenk (1939) showed that gangliosides accumulated in the brains of patients with familial infantile amaurotic idiocy. This increase has now been shown to be limited to only one special ganglioside, G_{M2}, later termed Tay-Sachs' ganglioside. This ganglioside has also been found in spleen and liver which suggests that Tay-Sachs' disease is a generalized disorder. In an allied disease, late infantile amaurotic idiocy, the normal major monosialoganglioside, G_{M1}, is increased. The nature of the enzymic lesions will be discussed.

The composition of gangliosides and glycolipids from post-mortem cases of infantile Gaucher's disease indicates that the neurological manifestations are caused by a disturbance in the metabolism of brain gangliosides. The minor gangliosides G_{M2} and G_{M3} were moderately increased and, to a larger extent, ceramide lactosides and glucocerebrosides accumulated with a similar ceramide composition as the gangliosides. These data fit a diminution of brain β-glucosidase activity, recently demonstrated (Brady, Kanfer & Shapiro, 1965) in spleen from patients with Gaucher's disease, leading to a disturbed catabolism of brain gangliosides.

Serious disturbances of the ganglioside pattern with a pronounced increase of the minor mono- and disialogangliosides G_2 and G_3 also occur in the leucodystrophies of the 'globoid cell' and metachromatic types, and in subacute leucoencephalitis. Also, in other inherited disorders, multiple sclerosis, and in presenile and senile dementia, the pattern of gangliosides can be changed. In some of these conditions, the concentration of a certain ganglioside was increased many-fold compared with normal values. However, the sequence of the carbohydrate moieties was the same, and the sphingosine and fatty acid composition showed no or only slight variations from the corresponding ganglioside of normal brains.

The physiological implications of the disturbances in the ganglioside pattern are still unknown.

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COMMUNICATIONS

A Study of Cerebral Sphingomyelins in Monomolecular Films

By J. H. RAPER, D. B. GAMMACK and G. H. SLOANE-STANLEY. (*Department of Biochemistry, Institute of Psychiatry, Maudsley Hospital, Denmark Hill, London, S.E. 5, and Department of Biochemistry, Institute of Basic Medical Sciences, Royal College of Surgeons of England, Lincoln's Inn Fields, London, W.C. 2*)

Sphingomyelins from ox brain have been separated into two fractions, one of which has as its major fatty acid, stearic acid (C18:0), the other fraction having almost exclusively nervonic acid (C24:1) (Eldin & Sloane-Stanley, 1964). Surface pressures were measured by the Wilhelmy plate method on monomolecular films of these fractions spread at an air–water interface.

The fraction containing predominantly stearic acid formed a liquid condensed film while the fraction enriched in nervonic acid formed a film of the liquid expanded type; the closest stable packings of these films were 40sq.Å/mol. and 56sq.Å/mol. respectively. The effects of unsaturation in the fatty acid and sphingosine constituents were then eliminated by hydrogenation, which converted the sphingosine to dihydrosphingosine and removed all unsaturation from the fatty acids, as shown by thin-layer chromatography. Hydrogenation of the stearoyl fraction had no significant effect on its surface pressure–area curve; on the other hand, hydrogenation of the nervonoyl fraction resulted in its forming a more condensed film, the surface pressure–area curve being now almost identical with that obtained for the stearoyl fraction.

The properties of a sphingomyelin monolayer appeared therefore to depend primarily on whether the predominant fatty acid was saturated or unsaturated, and only to a much smaller extent on whether the base was sphingosine or dihydrosphingosine. It has, indeed, already been shown that replacement of a saturated fatty acid in a lecithin by an unsaturated acid of the same hydrocarbon chain length results in the formation of a more expanded film (van Deenen, Houtsmuller, de Haas & Mulder, 1962).

The difference between the surface behaviour of the two fractions of native sphingomyelin may be related to the fact that, in the human brain, stearoyl sphingomyelin is largely confined to grey matter, while nervonoyl-sphingomyelin is a myelin lipid. (Svennerholm, 1963; Pilz & Jatzkewitz, 1964).

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The Role of the Polar Groups of Cerebral Sphingolipids on their Properties at the Air–Water Interface

By D. B. GAMMACK and J. H. RAPER. (*Department of Biochemistry, Institute of Psychiatry, Maudsley Hospital, Denmark Hill, London, S.E. 5*)

Cerebrosides, sulphatides and gangliosides, isolated in a high state of purity from ox brain, were spread as monomolecular films at the air–water interface. Surface pressure measurements were made on the films by the Wilhelmy plate method.

Cerebrosides and sulphatides produced liquid condensed films the surface pressure–area curves being coincident at pressures above 20dynes/cm. but at lower pressures the sulphatide films exhibited greater expansion than the cerebroside films. This difference is probably due to the sulphate group although an effect due to differences in the fatty acid composition, especially in the content of hydroxy acids (O'Brien, Fillerup & Mead, 1964), cannot be excluded.

Gangliosides formed films which were however highly expanded, the apparent minimum area per molecule for the ganglioside with one *N*-acetylneuraminic acid per sphingosine being 63sq.Å compared with 40sq.Å for the sphingomyelin fraction which had, like gangliosides, stearic acid as its predominant fatty acid (Raper, Gammack & Sloane-Stanley, 1966). Other fractions of ox brain gangliosides previously shown (Gammack, 1963) to have two or more neuraminic acid residues per

sphingosine gave even more expanded films. The greatly expanded nature of ganglioside films compared with those of cerebrosides, sulphatides and sphingomyelins is attributed to the much larger bulk of the polar end of the ganglioside molecule together with the effect of the anionic groups of the neuraminic acid residues.

Gangliosides and sulphatides spread on solutions containing up to 50mM-NaCl, -KCl or -$CaCl_2$ gave surface pressures which were not significantly different from those measured with distilled water as the hypophase. This contrasts with a marked condensation of phosphatidylserine monolayers in the presence of 1mM-$CaCl_2$, although 100mM-NaCl or -KCl had no measurable effect on the surface pressure–area curves (Rojas & Tobias, 1965). Thus the interactions of these cations with acidic groups of sugar residues in contrast to those of the less bulky polar groups of phospholipids do not markedly affect their packing in monolayers although such cations have been shown to change the state of aggregation of phospholipids and glycolipids in aqueous micellar dispersions (Gammack, 1963; Abramson, Katzman & Gregor, 1964, 1965).

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The Labelling of Brain Phospholipids by Cytidine 5′-Diphosphate Ethanolamine *in vitro*

By G. B. Ansell and R. F. Metcalfe. (*Department of Experimental Neuropharmacology, The Medical School, Birmingham*, 15)

There are approximately 16μmoles of ethanolamine plasmalogen, 7μmoles of phosphatidylethanolamine and 1·5μmoles of ethanolamine phospholipids containing alkoxy groups, per g. of whole rat brain (Ansell & Spanner, 1963; Horrocks & Ansell, 1965). On present evidence it seems likely that the ethanolamine moiety can be transferred to each of these lipids *in vivo* via Kennedy's cytidine pathway (Ansell & Spanner, 1965, and unpublished observations) although a pathway involving serine cannot be absolutely excluded (Wilson, Gibson & Udenfriend, 1960). McMurray (1964*a*,*b*) showed not only that ethanolamine-labelled cytidine diphosphate ethanolamine (CDP-ethanolamine) could serve as a precursor for these three lipids *in vitro*, but that the alkoxy analogue appeared to be labelled to a greater extent than the other ethanolamine lipids.

When CDP-ethanolamine (0·1μmole) labelled with [^{32}P]phosphorylethanolamine was incubated with 100mg. of a homogenate of adult rat brain, 20–25% of its radioactivity was incorporated into the phospholipids in 1hr. Of this incorporation, 40% was in phosphatidylethanolamine, 30% in the ethanolamine plasmalogen, 4% in a 'sphingomyelin' fraction and 3% in the ethanolamine alkoxy lipid. The remaining lipid labelling could not readily be accounted for. The time–specific radioactivity curves suggested that the three types of ethanolamine lipids were labelled independently.

The labelling of ethanolamine lipids in unit time varied with the age of the tissue. With brain tissue from a 23-day-old rat the rate was 43mμmoles of lipid/100mg. of tissue/hr. This was reduced to 23mμmoles with tissue from animals 70 days old. The peak for phosphatidylethanolamine labelling occurred at 15 days: 19mμmoles/100mg./hr. compared with 9mμmoles in the adult. The labelling of ethanolamine plasmalogen rose from 5mμmoles/100mg./hr. at 6 days to 12mμmoles in the 23-day-old rat, falling slowly to 6mμmoles in the adult. The maximum rate of labelling of this lipid *in vitro* fell within the period of its maximum rate of deposition *in vivo*. Only about 1mμmole/100mg./hr. of the alkoxy analogue of phosphatidylethanolamine was formed, whatever the age of the animal.

Transfer of [^{32}P]phosphorylethanolamine to a lipid will presumably depend on the amount of lipid acceptor present in the system used and the activity of the phosphotransferase present. Furthermore, the phosphotransferase reaction is reversible (Weiss, Smith & Kennedy, 1958; Bremer, 1965). The results will be discussed in relation to the biosynthesis of ethanolamine phospholipids in general.

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The Catabolism of Ethanolamine-Containing Phospholipids in Rat-Brain Preparations

By G. B. ANSELL and SHEILA SPANNER. (*Department of Experimental Neuropharmacology, The Medical School, Birmingham*, 15)

The presence of phospholipase A has been demonstrated in brain tissue by Gallai-Hatchard, Magee, Thompson & Webster (1962) using exogenous phosphatidylcholine and phosphatidylethanolamine from egg. Webster & Thompson (1965) have also shown that endogenous lecithin in the nervous system is degraded to lysolecithin. Apart from this phospholipase A and an enzyme hydrolysing the vinyl ether linkage of the ethanolamine plasmalogen (Ansell & Spanner, 1965), evidence for enzymes attacking ethanolamine phospholipids in brain is limited.

Previous findings (Ansell & Spanner, 1963, 1965) that there was a breakdown of the ethanolamine phospholipids when brain homogenates were incubated in Krebs bicarbonate Ringer, have now been extended. After 3hr. incubation the loss of lipid-bound ethanolamine (1·4 μmoles/g. brain/hr.) equalled the release of free ethanolamine. The breakdown was unaffected by the presence or absence of oxygen but was completely inhibited by EDTA. When pyruvate was added, the breakdown of the ethanolamine lipids and the release of free ethanolamine was reduced to 0·8 μmole and 0·9 μmole/g. brain/hr. respectively, suggesting the use of ethanolamine phospholipids as oxidizable substrates.

Loss of lipid phosphorus and liberation of free ethanolamine might be accounted for by the removal of fatty acids and aldehydes and the subsequent hydrolysis of glycerylphosphorylethanolamine. Alternatively, phosphorylethanolamine might be produced by phospholipase C action which could then be hydrolysed by phosphatase. To study this breakdown of ethanolamine phospholipids in more detail, the action of glycerol–bicarbonate extracts of rat-brain acetone powders on ethanolamine plasmalogen and phosphatidylethanolamine prepared from animal tissues was studied. Acyl esters, aldehydes and phosphate were liberated from the lipids by the extract but this effect was completely abolished by dialysing the extract. The addition of Mn^{2+} ions restored enzymic activity towards acyl ester groups. Zn^{2+} ions completely restored the activity towards the glycerol phosphate linkage, i.e. 1·0 μmole/g. fresh tissue/hr. for the plasmalogen and 1·8 μmoles/g. fresh tissue/hr. for the phosphatidylethanolamine. Acyl ester groups remained intact in the presence of Zn^{2+}. These observations would indicate the presence of a phospholipase C in brain, active towards ethanolamine lipids at neutral pH. Phosphatidylcholine was also broken down (1·2 μmoles/g. fresh tissue/hr.). This result may be compared with the findings of Roitman & Gatt (1963) who described an enzyme present in brain which split the glycerol phosphate linkage of choline lipids optimally at pH 4–5. It is also of some interest that Ottolenghi (1964) has found that Zn^{2+} ions activate phospholipase C in other tissues.

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Synthesis of Polyphosphoinositides by Rat Brain *in vitro*

By M. KAI and J. N. HAWTHORNE. (*Department of Medical Biochemistry and Pharmacology, The University, Birmingham*, 15)

The biosynthesis *in vitro* of diphosphoinositide from phosphatidylinositol was observed in a brain microsomal fraction by Colodzin & Kennedy (1964) and in rat-liver subcellular fractions by Michell & Hawthorne (1965).

The synthesis of diphosphoinositide from phosphatidylinositol and of triphosphoinositide from diphosphoinositide have been studied using rat-brain homogenate. The incubation medium was made up as follows: tris–HCl buffer, pH 7·4, 20mM; $MgCl_2$, 30mM; EDTA, 2mM; phosphatidylinositol, 1mM or diphosphoinositide, 0·9mM; terminally labelled [^{32}P]ATP, 5–6mM; brain homogenate (2mg. protein). The final volume was 1·0ml. After incubation at 37° for 2min. lipids were extracted and washed by a method similar to that of Garbus, DeLuca, Loomans & Strong (1963) and separated by chromatography on formaldehyde-treated paper (Kai & Hawthorne, 1966).

Among inorganic ions tested only Mg^{2+} and Mn^{2+} activated diphosphoinositide synthesis; Ca^{2+}, Cu^{2+}, K^+, Na^+, and F^- were inhibitory. Ouabain (1mM) had no effect in the presence of Na^+ and K^+, while acetylcholine (10^{-5} and 10^{-4}M) with eserine (10^{-4}M) stimulated the synthesis of diphosphoinositide (cf. Santiago-Calvo, Mulé, Redman, Hokin & Hokin, 1964). Deoxycholate (3mM) activated the enzyme more effectively than other detergents. There was no obvious relation between the effects of various detergents and their ionic charges.

Subcellular fractionation studies showed that nuclear, nerve-ending and microsomal fractions were more active in diphosphoinositide synthesis. These fractions also possessed most Na^+–K^+-stimulated ATPase activity (Hosie, 1965). On the other hand the synthesis of triphosphoinositide was found to be most active in the supernatant fraction. The distribution of 5′-mononucleotidase did not show a definite correlation with polyphosphoinositide synthesis (cf. Michell & Hawthorne, 1965).

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Phospholipids and Cholesterol in Particulate Fractions of Adrenal Medulla

By H. Blaschko, H. Firemark, A. D. Smith and H. Winkler. (*Department of Pharmacology, University of Oxford*)

Chromaffin granules, mitochondria and microsomes were obtained from homogenates of bovine adrenal medulla by differential and sucrose density gradient centrifugation. The lipids were extracted by the method of Folch, Lees & Sloane-Stanley (1957). Cholesterol determinations were carried out on the extracts (Zlatkis, Zak & Boyle, 1953) and the phospholipids were separated by thin-layer chromatography and estimated as described by Skipski, Peterson & Barclay (1964).

The molar ratio of total cholesterol/lipid P was higher in the chromaffin granules (0·7) than in the microsomes (0·5) or in the mitochondria (0·3). In other words, the lipids of the chromaffin granules are rich in cholesterol.

The phospholipids were provisionally identified by comparing their chromatographic mobilities with reference compounds, and by their staining reactions with ninhydrin and Dragendorff's reagent. Phospholipids containing ethanolamine, serine, choline and sphingosine were present in each of the three particulate fractions.

Lysolecithin was present in relatively high concentration in chromaffin granules: 16% of the total lipid P of this fraction was in lysolecithin, as compared with 2% in both the microsomes and the mitochondria. Lysolecithin in the chromaffin granules was further characterized by its ability to lyse human erythrocytes. The lecithin content of the chromaffin granules was rather low when compared with that of the other two fractions.

The remarkably high lysolecithin content of chromaffin granules is of interest in view of the finding by Feldberg (1940) that lysolecithin is a potent releaser of adrenaline from the perfused adrenal gland of the cat.

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Preparation of 1,2-Di-*O*-Acylglycerols

By Jill Gigg and R. Gigg. (*National Institute for Medical Research, Mill Hill, London, N.W.* 7)

1,2-Di-*O*-acylglycerols are of interest in both the chemical and biochemical syntheses of phospholipids. 1,2-Di-*O*-acyl-L-glycerols (in which the acyl groups are saturated) are readily prepared by hydrogenolysis of the benzyl group in 1,2-di-*O*-acyl-3-*O*-benzyl-L-glycerols and the method can be extended to a preparation of the unsaturated compounds if the double bonds are protected by bromination (Baer & Buchnea, 1958; Buchnea & Baer, 1960). Recently a convenient preparation of racemic 1,2-di-*O*-oleoylglycerol has been described (Krabisch & Borgström, 1965) in which 1,2-di-*O*-oleoyl-3-*O*-2′-tetrahydropyranylglycerol was hydrolysed by acid, under conditions which did not cause isomerization of the product to 1,3-di-*O*-oleoylglycerol. The method was limited to the preparation of racemic compounds since the 1-*O*-2′-tetrahydropyranylglycerol used was prepared by the hydroxylation of allyl tetrahydropyranyl ether.

We have recently prepared racemic glycerol 1,2-carbonate (Cunningham & Gigg, 1965) and have now used it for the preparation of 3-*O*-2′-tetrahydropyranylglycerol 1,2-carbonate which gives racemic 1-*O*-2′-tetrahydropyranylglycerol on alkaline hydrolysis. We have also prepared racemic 1-*O*-1′-ethoxyethylglycerol from the reaction of ethyl vinyl ether with glycerol 1,2-carbonate followed by alkaline hydrolysis. The di-*O*-palmitoyl

esters of these two compounds give racemic 1,2-di-*O*-palmitoylglycerol on acid hydrolysis under the conditions described (Krabisch & Borgström, 1965), the ethoxyethyl ether being hydrolysed more rapidly than the tetrahydropyranyl ether. The tetrahydropyranyl and ethoxyethyl groups are also removed from these compounds by the boric acid method (Martin, 1953; Hartman, 1959) to give 1,2-di-*O*-palmitoylglycerol and this procedure is preferred since isomerization to 1,3-di-*O*-palmitoylglycerol does not occur.

We have also prepared L-glycerol 1,2-carbonate from L-3-*O*-benzylglycerol by the method described for the racemic compound (Cunningham & Gigg, 1965) and have demonstrated its optical purity by conversion to the enantiomer of the previously described 1-*O*-trityl-L-glycerol (Baer & Fischer, 1945). L-Glycerol 1,2-carbonate should therefore be a suitable intermediate for the preparation of unsaturated 1,2-di-*O*-acyl-L-glycerols using the routes outlined above. By acylation of 1-*O*-tetrahydropyranyl-3-*O*-trityl-D-glycerol followed by hydrogenolysis of the trityl group and re-acylation with a different acyl chloride, a new route to the 1,2-di-*O*-acyl-L-glycerols containing different acyl groups should be possible.

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The Formation of Δ^{16}-Steroids *in vitro* from 3β-Hydroxy-pregn-5-ene-20-one by Rat Testis Tissue

By N. Ahmad and D. B. Gower. (*Department of Biochemistry and Chemistry, Guy's Hospital Medical School, London, S.E.* 1)

The metabolism of pregn-5-en-3β-ol-20-one (pregnenolone) by testicular tissue has been studied in various species but the biosynthesis of Δ^{16}-steroids from pregnenolone *in vitro* has not hitherto been reported.

Minced rat testis tissue was incubated with [4-^{14}C]-pregnenolone for 4 hr. in Krebs–Ringer bicarbonate–glucose medium. Each flask also contained 1 μmole each of NAD, NADP and ATP. After incubation, steroids were extracted with ethyl acetate, the extracts chromatographed on alumina and Δ^{16}-compounds eluted with light petroleum – benzene (1:1, v/v) essentially as described by Gower & Haslewood (1961). Steroids more polar than these were eluted with benzene–ethanol mixtures. Fractions containing Δ^{16}-compounds were submitted to thin-layer chromatography against authentic marker steroids as described by Gower (1964). Radioautography revealed radioactive spots corresponding to the mobility of androsta-4,16-dien-3-one and 5β-androst-16-en-3α-ol. Confirmation of the identity of these compounds was obtained by re-chromatography in several systems and preparation of the 2,4-dinitrophenylhydrazone and the acetate but the small quantity of authentic steroids available was not sufficient for recrystallization. Investigation of steroids more polar than the Δ^{16}-compounds by paper and thin-layer chromatography in several systems and by recrystallization to constant specific activity (where possible) revealed the presence of androst-4-ene-3,17-dione, 17α-hydroxypregnenolone, progesterone, 17α-hydroxyprogesterone and testosterone, confirming the results of Shikita, Kakazaki & Tamaoki (1964). In addition, labelled dehydroepiandrosterone and small amounts of 16α-hydroxyprogesterone and of androst-5-ene-3β,17β-diol were found, the last-mentioned compound being a metabolite of pregnenolone which has not previously been reported in rat testis. The possible pathways of the biosynthesis of the Δ^{16}-compounds will be discussed.

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Presence of Allocholic Acid in Mammalian (including Human) Bile

By A. R. Tammar (introduced by G. A. D. Haslewood). (*Guy's Hospital Medical School, London, S.E.* 1)

Allocholic acid has been found in the biles of a variety of non-mammalian vertebrates, generally in association with cholic acid. It has now been found in twelve mammalian species, including man. Ethyl allocholate derived from the bile of these species was detected by thin-layer chromatography (TLC) on silica gel, with the system diethyl oxalate–dioxan (7:3, v/v). In this system ethyl allocholate runs a little slower than ethyl cholate. Alumina column fractions of bile acid ethyl esters from 40 species of mammals were screened using this method and in 12 cases (**Macaca irus*, *Myrmecophaga tridactyla*, **Herpestes edwardsi*, **Puma concolor*, *Zalophus californianus*, **Orycteropus afer*, *Ovis musimon*,

**Damaliscus albifrons*, **Ourebia ouribi*, *Lithocranius walleri*, *Oryx beisa*, *man) the double spot typical of ethyl cholate–ethyl allocholate mixtures was seen. The relevant fractions were crystallized from benzene to yield material with the properties of the ethyl ester of 'Ohta's acid', i.e. a mixture of allocholic acid and cholic acid (2:1, w/w) (Anderson & Haslewood, 1962). Infrared spectra were obtained in the seven cases* where sufficient material was available and these showed the characteristic bands of both cholic acid and allocholic acid esters.

Allocholic acid was present only in very small quantities in the mammalian species in which it was found. As an example of its identification: 335ml. of human fistula bile yielded dry bile salts (4·5g.). This material (4·0g) was hydrolysed, and the product esterified, yielding bile acid ethyl esters (1·8g.). These were separated on an alumina column into 16 fractions of which 5 (total wt., 407mg.) had the properties of esters of trihydroxy bile acids. Of these, one fraction (54mg.) was shown on TLC possibly to contain ethyl allocholate. Two crystallizations of this fraction from benzene yielded needles (3·9mg.), m.p. 199–201°, the infrared spectrum of which was identical with that of a 2:1 (w/w) mixture of ethyl allocholate and ethyl cholate.

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Formation of Δ^3-Isopentenyl Phosphate by *Hevea brasiliensis* Latex

By C. J. Chesterton and R. G. O. Kekwick. (*Department of Biochemistry, The University, Birmingham*, 15)

In addition to the formation of substances having the characteristics of 5-phosphomevalonate, 5-pyrophosphomevalonate and Δ^3-isopentenyl pyrophosphate on paper chromatography in butan-1-ol–formic acid–water (77:13:10, by vol.; solvent 1) from [2-^{14}C]mevalonate by *Hevea* latex, Archer, Audley, Cockbain & McSweeney (1963) have also reported the formation of an unidentified substance having R_F 0·13.

We also have observed the formation of an unidentified substance by latex from [2-^{14}C]mevalonate having similar chromatographic characteristics to those described by Archer *et al.* (1963).

When protein-free mixtures obtained by incubating latex (0·1ml.) with [2-^{14}C]mevalonate (0·5 μmole, 0·05μc) and ATP (1μmole) for 30min. at 30° were applied to a Dowex 1 column, and were eluted with a gradient of increasing concentration of formate ion according to the procedure of Bloch, Chaykin, Phillips & de Waard (1959), radioactivity separated in three fractions in addition to those which could be ascribed to mevalonic acid and its lactone. Fraction I was identified as 5-phosphomevalonate, the formation of which in latex has been described by Williamson & Kekwick (1965). Fraction II did not correspond to any compound hitherto reported and fraction III had the chromatographic characteristics of Δ^3-isopentenyl pyrophosphate. Very little radioactivity was eluted in the conditions described by Bloch *et al.* (1959) for the elution of 5-pyrophosphomevalonate. When latex serum was incubated with [1-^{14}C]mevalonate only fraction I contained radioactivity. Incubations carried out for different times showed a precursor–product relation between fractions I and III and fractions III and II.

Paper chromatography, of protein-free material derived from similar incubation mixtures, in ethanol–ammonia–water (7:2:1, by vol.) (solvent 2), gave three components in addition to mevalonate. When these components were applied to the Dowex 1 column the fraction A having R_F 0·13–0·17 was eluted as fraction I, B having R_F 0·2–0·23 as fraction III and C having R_F 0·4–0·5 as fraction II.

Freeze-dried latex serum was reconstituted and dialysed against phosphate buffer, pH 7·5, and 1ml. of this containing 10mg. of protein was incubated with [2-^{14}C]mevalonate (5μmoles, 0·5μc) AT^{32}P (10μmoles, 3·2μc) and $MgCl_2$ for 30min. at 30°. Chromatography of the protein-free material from this incubation in solvent 2 gave rise to the three fractions containing both ^{14}C and ^{32}P. The atomic ratios of ^{14}C to ^{32}P in these fractions were: fraction A, 1:1·11; fraction B, 1:2·0; fraction C, 1:1·06.

When the three radioactive fractions were prepared by incubating reconstituted freeze-dried latex serum with [2-^{14}C]mevalonate followed by paper chromatography in solvent 2, fractions A and B were incorporated almost completely into rubber and fraction C was not.

Fraction C, on chromatography in solvent 2, gave a spot coincident with chemically synthesized Δ^3-isopentenyl phosphate kindly given by Dr D. Barnard.

Removal of the large particulate fraction of latex which contains phosphatase (Archer *et al.* 1963) by centrifuging at 2000*g* for 20min. gave a supernatant which formed only fractions I and III from [2-^{14}C]mevalonate. From this it is concluded that the unknown compound in fraction II is Δ^3-isopentenyl phosphate formed from Δ^3-isopentenyl pyrophosphate by the action of a phosphatase present in the large particulate fraction of latex.

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Phosphomevalonate Kinase from *Hevea brasiliensis* Latex

By D. N. Skilleter, I. P. Williamson, and R. G. O. Kekwick. (*Department of Biochemistry, The University, Birmingham, 15*)

Although very little 5-pyrophosphomevalonate appears to accumulate in latex during the formation of isopentenyl pyrophosphate from mevalonate, it has been found possible to obtain, from reconstituted freeze-dried latex serum, a preparation of phosphomevalonate kinase (free of pyrophosphomevalonate decarboxylase) which catalyses the phosphorylation of 5-phosphomevalonate to 5-pyrophosphomevalonate in the presence of ATP.

Freeze-dried latex serum prepared by the method of Archer & Sekhar (1955) was reconstituted with water to its original concentration, and was dialysed against phosphate buffer, pH 7·0, 50mM, containing $MgCl_2$ (5mM) and *N*-acetylcysteine (4mM).

The dialysed solution (3ml. 100mg. protein) was applied to a Sephadex G-200 column (25cm. × 2cm.) and was eluted with the phosphate buffer giving two protein fractions. The fraction retarded by the column contained enzymes which were shown by chromatography of their reaction products to convert 5-phospho[2-^{14}C]mevalonate to isopentenyl pyrophosphate. This active protein fraction was concentrated by pressure dialysis and was applied to a DEAE-Sephadex column (12 × 1·3cm.) which had been equilibrated with the phosphate buffer. Elution of the protein from the column with the phosphate buffer containing KCl, the concentration of which increased in a gradient to 0·6M, produced two active fractions. The protein fraction eluted at low concentrations of KCl contained both phosphomevalonate kinase and 5-pyrophosphomevalonate decarboxylase, but the protein fraction eluted at concentrations of KCl between 0·1 and 0·25M contained phosphomevalonate kinase separated from 5-pyrophosphomevalonate decarboxylase. 5-Pyrophosphomevalonate was the sole product, identified by chromatography on Dowex 1 by the procedure of Bloch, Chaykin, Phillips & de Waard (1959) and on paper, formed by the action of this fraction on 5-phospho[2-^{14}C]mevalonate. The fraction catalysed the phosphorylation of 0·1 μmole of 5-phosphomevalonate/min./mg. of protein as estimated by the production of ADP assayed by the method of Lynen (1959), as modified by Williamson & Kekwick (1965).

Phosphomevalonate kinase from *Hevea* latex serum appears to be a fairly unstable enzyme. Activity is optimum between pH 7·0 and pH 7·5 and is lost irreversibly when the pH of the enzyme solution is outside this range. The activity of the enzyme preparation was maximal at 40° and decreased sharply at higher temperatures. There was a requirement for either Mg^{2+} or Mn^{2+} ions and for thiol compounds, activity being irreversibly lost when these substances are removed from the medium. The concentration of Mg^{2+} giving maximal activity was 8mM, the activity obtained being about 2·5 times that given in the presence of the optimal concentration of Mn^{2+} which was 3mM. The K_m values at 30° were 0·04mM-5-phosphomevalonate and 0·14mM-ATP, both of these values increased with increasing temperatures.

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Inhomogeneity of Lysolecithin-Solubilized Membrane Systems

By W. L. G. Gent and N. A. Gregson. (*Biochemistry and Chemistry Department, Guy's Hospital Medical School, London, S.E. 1*)

Rat-brain myelin solubilized by lysolecithin has been shown (Gent, Gregson, Gammack & Raper, 1964) to behave as a single-component system at pH 8·6, *I* 0·1, both in the Tiselius electrophoretic cell and in the analytical ultracentrifuge. It has been found possible now to demonstrate the presence of three components electrophoretically at this pH by (*a*) solubilization with the minimal quantity (w_{min}) of lysolecithin required for maximal solubilisation; (*b*) electrophoresis in buffer of *I* 0·1 or less; (*c*) electrophoresis at 25°.

Dialysis of myelin solubilized with w_{min} of lysolecithin against buffers of various pH values results in complete precipitation in the range pH 7·5–6·5. Below pH 6·5 up to 50% of the material is resolubilized and on electrophoresis at pH 8·6, *I* 0·1, the soluble fraction shows three components again. By solubilization with $2 \times w_{min}$ of lysolecithin

it has been found that precipitation is avoidable below pH 6 and such solutions show two major electrophoretic components, the smaller (C) having an isoelectric pH of 5·8 and the larger (B) an isoelectric pH of 5·5. The electrophoretic separation is well-marked at pH 4·0, I 0·1, when for B the mobility equals $1·3 \times 10^{-5}$ and for C the mobility equals $4·2 \times 10^{-5}$. Ultracentrifugation of the solution indicated two components of which the smaller has the higher sedimentation constant. Fractionation of the solution on Sephadex G-200 columns confirmed that the fraction C is of higher mol.wt. (greater than 250000) and that fraction B is diffuse with mol.wt. in the range 150000–200000. Isolation and analysis of the two fractions indicated that the total lipid/lysolecithin ratio = 1:1 in C and 1:3 in B. Both fractions contained the complete range of native lipids but the proportion of cholesterol in C is greater than in B.

A similar range of experiments has been made on solutions of human red-cell stroma solubilized with lysolecithin and has yielded analogous results. Dogfish myelin, on the other hand, although showing two electrophoretic components at pH 8·6 but one ultracentrifugal component, is unaltered at pH 4·0.

It is concluded that mammalian membrane systems probably contain at least two components as the major structural elements.

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Entry of Ethionine Sulphur into Yeast Protein

By G. A. Maw. (*Arthur Guinness Son & Co. Ltd., St. James's Gate, Dublin, Ireland*)

Ethionine is known to replace methionine residues in the proteins of a number of organisms (Gross & Tarver, 1955; Rabinovitz, Olson & Greenberg, 1957; Yoshida & Yamasaki, 1959; Hansson & Garzo, 1962). In yeast, ethionine has been shown to be taken up and the sulphur of the amino acid accumulated by the cells (Maw, 1963*a*), although the form in which this sulphur was retained was not established.

The present studies have been carried out to determine the extent of incorporation of ethionine-sulphur into the protein fraction of *Saccharomyces cerevisiae* and *Torulopsis utilis*, also the form in which the sulphur becomes bound in the protein. Ethionine-sensitive and ethionine-resistant strains of these two yeasts were grown in the presence of 0·78 mM-L-[^{35}S]ethionine and after 2 days the cells were harvested and labelled sulphur uptake determined. The trichloroacetate-insoluble fractions of cells were subjected to performic acid oxidation, followed by acid hydrolysis (Maw, 1963*b*, 1965), and chromatography of the products in *n*-butanol–acetic acid–water (12:3:5, by vol.) carried out. Examination of the resulting radiochromatograms revealed the presence of [^{35}S]ethionine (as its sulphone) in amounts corresponding to a replacement of 21% of the protein methionine of *S. cerevisiae* and 12% of that of *T. utilis*.

In addition, significant amounts of labelled cysteic acid and methionine sulphone were identified, indicating not only the incorporation of ethionine *per se*, but also a sulphur transfer from ethionine to cyst(e)ine and methionine. The amount of labelled sulphur present as cystine + methionine in the ethionine-sensitive strains was some 15% of the total ethionine sulphur incorporated into the protein of both yeasts (5–6% as cystine, 9% as methionine), and this value increased to nearly 50% (18–19% as cystine, 27–29% as methionine) after the yeasts had been allowed to grow for 7 days in the presence of labelled ethionine. There was a more extensive breakdown of ethionine in the ethionine-resistant strains. Thus in *T. utilis* grown for 7 days under these conditions, only 10% of the labelled sulphur incorporated was identified as ethionine itself, 38% being present as cystine and 52% as methionine.

The most likely mechanism for this sulphur transfer is via the initial formation of *S*-adenosylethionine, a reaction established previously by Parks (1958), followed by de-ethylation of this compound and fission of the resulting *S*-adenosylhomocysteine to homocysteine. The latter compound would then undergo transsulphuration and methylation by known pathways, leading to cyst(e)ine and methionine respectively. Ethionine resistance in these organisms appears to result from an increased ability to degrade ethionine, coupled with a diminished uptake of the amino acid by the cells.

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Role of *p*-Hydroxybenzoic Acid in the Metabolism of *Escherichia coli*

By R. G. WYN JONES and J. LASCELLES.* (*Microbiology Unit, Department of Biochemistry, University of Oxford*)

It has recently been suggested that *p*-hydroxybenzoic acid (*p*-OB) is an intermediate in the biosynthesis of ubiquinone in micro-organisms and mammalian tissues (Parsons & Rudney, 1964, 1965; Cox & Gibson, 1964; Olsen, Dialameh, Bentley, Springer & Ramsey, 1965). Multiple aromatic mutants of *Escherichia coli* have been isolated which require *p*-OB, in addition to phenylalanine, tyrosine, tryptophan and *p*-aminobenzoic acid, for rapid growth (Davis, 1952). A mixture of lysine and methionine largely replaced *p*-OB in stimulating rapid growth, and *p*-OB was suggested to be involved in the biosynthesis of these amino acids (Davis, 1955).

The metabolic role of *p*-OB has been studied using *E. coli* 156:53D2, an auxotroph requiring *p*-OB in addition to the other aromatic metabolites for rapid aerobic growth. No requirement for *p*-OB was found anaerobically. A limited stimulation of aerobic growth in the absence of *p*-OB was observed with lysine and methionine and with succinate. The action of lysine and methionine is attributed to a sparing of succinyl-CoA which is involved in their biosynthesis.

Wild-type *E. coli* 518 grown aerobically contained both ubiquinone (0·3 μmole/g. dry wt. of cells) and vitamin K_2 (0·1 μmole/g. dry wt.) The auxotroph grown aerobically in the presence of *p*-OB contained ubiquinone (0·7 μmole/g. dry wt.) but little vitamin K_2 ($<0{\cdot}01$ μmole/g. dry wt.), whereas neither quinone was detected in the absence of *p*-OB. Both wild-type and auxotroph organisms had diminished quinone contents when grown anaerobically. The mutant grown aerobically without *p*-OB was unable to respire with glucose or succinate, and extracts showed decreased $NADH_2$-oxidase and succinate-oxidase activities. Quinones have previously been implicated in the $NADH_2$- and succinate-oxidase complexes of *E. coli* (Kashket & Brodie, 1963).

Low α-oxoglutarate-dehydrogenase activity was found in organisms grown aerobically without *p*-OB, compared with those grown with *p*-OB. The stimulation of *p*-OB-deficient growth by succinate is attributed to a decreased capacity of aerobic quinone-deficient cells to synthesize succinate. Anaerobically, succinate is formed via fumarate reductase which is repressed by oxygen.

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Possible Hydrophobic Bonding in Sulphatase A

By A. B. ROY* and L. W. NICHOL. (*Department of Physical Biochemistry, Australian National University, Canberra*)

Sulphatase A of ox liver exists as a monomer (mol.wt. 107 000) above pH 6·5 and as a tetramer (mol.wt. 411000) below pH 5·5. Previous attempts to identify the bonding in the tetramer have failed but electrostatic linkages and the more common covalent bonds have been excluded. It therefore seemed probable that hydrogen and/or hydrophobic bonds were involved, the latter being more likely because the former are essentially electrostatic.

At pH5 the $\bar{S}_{20,w}$ of the tetramer increased from 13·61 to 14·23s as the temperature increased from 1° to 38°: this suggested a hydrophobically bonded structure being strengthened by the rise in temperature. The $\bar{S}_{20,w}$ also increased as the pH was taken towards the isoelectric point of 3·4; this again was consistent with the strengthening of hydrophobic bonding as the net charge on the molecule decreased.

The action of sodium dodecyl sulphate on sulphatase A has been investigated. At pH 5 a complex having an S_{20} of 3·6s was produced to an extent dependent upon the detergent/protein ratio. Conversion was complete when this ratio was 550. The 3·6s material was shown to be homogeneous with respect to S by boundary analyses from which D was computed. The S/D ratio so obtained gave a molecular weight of 57000, a value confirmed by equilibrium centrifugation. If the complex contained detergent and protein in the above ratio then the molecular weight of its protein component was

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24 000, suggesting that the detergent had split the tetramer into quarter subunits of the monomer.

It is therefore postulated that the association of sulphatase A is governed by the net charge on the molecule but that the actual binding of monomer to form tetramer is principally by hydrophobic bonds. The polymerization is therefore essentially a means of preventing the decrease in entropy associated with the changes in the water structure caused by non-polar groups in the protein: these groups themselves associate provided that the electrostatic repulsive forces are not too great. On extreme dilution the entropy sacrifice in retaining the tetramer would become too great and its dissociation would be expected, as has indeed been found. The subunits of the monomer are probably also hydrophobically bonded but this has not yet been shown unambiguously.

Multiplicity of Liver Uridine 5′-Pyrophosphate Glucuronyltransferase from its Varying Pattern of Development with Different Substrates and Species

By G. J. Dutton and Jane Lawes. (*Biochemistry Department, Queen's College, Dundee*)

Circumstantial evidence accumulates (see Storey, 1965; Dutton, 1964, 1965) for the multiplicity of liver-microsomal UDP-glucuronyltransferase (GT) which, developing perinatally, catalyses glucuronide formation from UDP-glucuronic acid.

If several GT's exist, they might develop differently towards different substrates and among different species. A clear demonstration of such differential development would, besides supporting multiplicity, also help to answer a question important in perinatal studies on glucuronide synthesis: how generally applicable are results obtained with any one substrate in any one species?

Fragmentary evidence exists both for and against a fairly general application, but the present work demonstrates clearly dissimilar development of GT activity towards three phenolic substrates; and that these rates themselves differ markedly between mouse and rat. Presented graphically, these detailed results appear conclusive.

GT activity was followed in perinatal mouse- and rat-liver homogenates and compared (protein-N basis) with that in maternal preparations (which exhibited consistent levels). Conjugates formed were demonstrably glucuronides and two substrates were usually compared simultaneously. Donor or acceptor concentrations were not limiting.

Considering two substrates in one species: in foetal mice, GT activity was always lower for *o*-aminophenol than for phenolphthalein; e.g., mean ratios were 10:40, 15:42, 20:87 (maternal values 100) at 16, 17 and 18 days' gestation respectively. After birth, the distinction faded. Molar ratios of substrate conjugated confirmed this pattern.

In perinatal rats, activity towards phenolphthalein was much lower than towards *p*-nitro phenol. Expressed as above, mean ratios were 15:140, 30:160, 45:170 (maternal values 100) for late foetuses, 1- and 2-day infants respectively, approaching 100:100 by 5–6 days. Mean molar ratios of conjugated substrate were 2:2 (maternal), 14:6 (1–3 days).

Considering each substrate in the two species (cf. Dutton, Langelaan & Ross, 1964): approximate percentage of maternal-liver GT possessed by mouse and rat liver respectively ($\pm$ 12 hours of birth) were: *o*-aminophenol, 20 and 150; *p*-nitrophenol, 70 and 200; phenolphthalein, 86 and 20. No inhibitors appeared; e.g., newborn rat-liver homogenate did not inhibit phenolphthalein glucuronide synthesis in foetal mouse-liver homogenates. As the patterns occurred also in sliced tissue, the differences presumably persist *in vivo*.

The conclusion that appearance of GT activity must be determined separately for substrate and species is of obvious importance in perinatal pharmacology.

The simplest explanation is that several GT's exist in each species, their development being characteristic of the species. But developing microsomes might selectively accept various substrates. Though unlikely because of the striking species differences observed, this possibility necessitates work with a solubilized GT (Isselbacher, Chrabas & Quinn, 1962) more stable than yet obtained.

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Formation of Glycine from L-[U-^{14}C]Glutamine in Guinea-Pig-Liver Slices

By D. F. Evered and Militsa Wass. (*Department of Chemistry, Chelsea College of Science and Technology, Chelsea, London, S.W.* 3)

Glycine conjugates with benzoic acid to form hippuric acid in mammalian liver. However,

endogenous glycine in rat liver and blood plasma is only sufficient for conjugation of very small amounts of benzoate (Arnstein & Neuberger, 1951). The principal source of glycine in rats and guinea pigs is serine (Shemin, 1946).

Unlabelled glutamine gave a greater stimulation of hippuric acid synthesis than did glutamic acid with guinea-pig-liver slices. Direct cleavage of glutamine to yield glycine and a 3-carbon compound was assumed (Leuthardt, 1941). Conversely, isotope experiments were compatible with an indirect conversion of glutamic acid to glycine via the tricarboxylic acid cycle. Glutamine itself was not tested (Shemin, 1949).

The reverse reaction, [2-^{14}C]glycine→glutamine, was studied in guinea-pig-liver slices. Radioautography of paper chromatograms suggested the presence of labelled glutamic acid in hydrolysed incubation mixtures (D. F. Evered & F. Leuthardt, unpublished experiments). Stepwise degradation of such labelled glutamic acid resulted in a possible reaction sequence (Jenny & Leuthardt, 1961). Reversal of this sequence for the conversion of glutamine to glycine is unlikely since the reaction catalysed by L-serine dehydratase is practically irreversible (Jenny & Leuthardt, 1961).

Guinea-pig-liver slices were incubated with benzoate, L-[U-^{14}C]glutamine and carrier L-glutamine. Total glycine was isolated and determined as DNP-glycine using buffered Celite columns (Krol, 1952). This DNP-glycine was found to be labelled with ^{14}C. The DNP derivatives of alanine, valine, glutamine and glutamic acid did not interfere with the separation of DNP-glycine on these columns.

Our results confirm that glutamine can give rise to the glycine moiety of hippuric acid in guinea-pig-liver slices. Conversion of unlabelled glutamine to glycine occurs in rabbits and guinea pigs but not in rats (Leuthardt, 1941). Therefore, serine formation from L-glutamine and hydroxypyruvate observed in rat-liver homogenates (Rowsell, Carnie & Wahbi, 1965) cannot be ascribed to intermediary formation of glycine.

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Kinetics of the Inhibition of Carbonic Anhydrase by Sulphonamides

By J. C. KERNOHAN. (*Department of Colloid Science, Cambridge University*)

Carbonic anhydrase is specifically inhibited by aromatic compounds having an unsubstituted sulphonamide group. Two reports (Davis, 1959; Leibman, Alford, & Boudet, 1961) on the kinetics of this inhibition agree that it is non-competitive with respect to carbon dioxide. Unfortunately in neither of these reports were the rates of the association and dissociation reactions involving the enzyme and inhibitor fully considered. Recently I have devised a technique for the measurement of the rates of these reactions. The rates are sufficiently low to invalidate the assumption made by previous workers that the enzyme–inhibitor system reaches equilibrium during the short time-interval in which it is in contact with CO_2 and in which the activity of the enzyme must be measured. Thus the enzyme inhibitor system is 'frozen' during the assay and the inhibition observed is independent of the substrate concentration. If this kinetic effect is taken into account then the inhibition by sulphonamides appears to be competitive.

Carbonic anhydrase in imidazole buffer, pH 7·7, containing *p*-nitrophenol indicator was mixed with CO_2 solutions containing various concentrations of benzenesulphonamide in a spectrophotometric stopped-flow apparatus and the hydration followed in the interval, about 0·1 sec. before the enzyme was completely inhibited. From the observed course of the reaction the rate constant for the combination of the enzyme with inhibitor may be calculated. At low initial CO_2 concentration, 1·6 mM, the rate constant for the reaction at 25° was $1{\cdot}57 \times 10^5$ M^{-1}sec.$^{-1}$. At the highest initial CO_2 concentration employed, 16·5 mM, the rate constant was $8{\cdot}5 \times 10^4$. Assuming a value near 12·5 mM for the K_m for CO_2 under these conditions (Kernohan, 1964) these values are consistent with the inhibition being competitive. The change in rate constant cannot be ascribed to the change in pH during the reaction as the amount of CO_2 hydrated by the time that the enzyme was completely inhibited was similar in each experiment.

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Nucleic Acids of Senescent Bean Leaves

By A. Wood and J. W. Bradbeer. (*Botany Departments, University College of Wales, Aberystwyth and King's College, University of London*)

Senescence of the primary leaves of Canadian Wonder Bean (*Phaseolus vulgaris* L.) was induced by excising the shoot and standing it in distilled water under 16hr.-long days at 21°. Rooting of the shoot, which would induce a recovery from leaf senescence, was prevented by daily removal of the lowermost 1–2cm. of the stem. RNA was extracted from acetone powders of the leaves by an alkaline hydrolysis procedure based on that of Nieman & Poulsen (1963) with some modifications suggested by Millikan & Pickett (1964) and Dr D. P. Holdgate. The RNA content of the leaves, based on the extinction of the purified nucleotides at 260 and 280mμ, fell from 995 to 393μg./g. fresh wt. over 6 days. At the same time the chlorophyll content of the leaves fell from 1·99 to 1·04mg./g. fresh wt.

Extraction of undegraded nucleic acids from senescent leaves by a phenol method required the inclusion of bentonite in the extraction medium. Bentonite prevented hydrolysis of the RNA by leaf ribonuclease during the extraction and the subsequent fractionation of the nucleic acids on a methylated serum albumin-kieselguhr column. The column eluate contained four main fractions, s-RNA, a DNA fraction containing RNA and light and heavy ribosomes. Total RNA, obtained by summing the s-RNA and ribosomal RNA fractions, showed a fall from 408 to 175μg./g. fresh wt. over the first three days, subsequently falling to 118μg. after 9 days' senescence. During these 9 days the s-RNA, DNA–RNA fraction and ribosomal RNA respectively fell to 42, 30 and 26% of the amounts originally present.

It may be noted that the total RNA contents of the leaves, obtained by summing the s-RNA and ribosomal RNA values, were much lower than the values found by the alkaline hydrolysis procedure. These discrepancies are thought to have resulted mainly from the interference of other substances in the alkaline hydrolysis method.

With this modified procedure for the extraction and fractionation of undegraded nucleic acids, a fresh approach to the investigation of nucleic acid metabolism in senescent leaves is made possible.

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The Breakdown of Adenosine Diphosphate and of Adenosine Triphosphate in Plasma

By D. C. B. Mills. (*Department of Pharmacology, Royal College of Surgeons of England, Lincoln's Inn Fields, London, W.C. 2*)

The reversibility of the aggregation by ADP of blood platelets in platelet-rich plasma (Born, 1962) suggests the presence in plasma of enzymes capable of destroying ADP. Hitherto no such enzyme has been described. It has been shown (Ireland & Mills, 1964) that ADP added to plasma is quickly degraded to AMP and to adenosine, but the experiments did not indicate whether the phosphate was released as inorganic orthophosphate (P_i) or was transferred to an acceptor. Jørgensen (1956) suggested a phosphate transfer reaction to account for his observation that ATP incubated in plasma disappeared but gave rise to less than the expected amount of P_i. The missing phosphate has now been identified as pyrophosphate (PP_i) and the direct dephosphorylation of ADP has been demonstrated.

Human plasma, prepared carefully to minimize haemolysis, was incubated with [γ-^{32}P]ATP, and the perchloric acid-soluble products were separated by paper electrophoresis (0·05M-citrate, pH3·8; 25v/cm. for 2hr. at 4°) with PP_i, P_i, ATP, ADP and AMP added as carriers. These compounds were detected after acid hydrolysis by spraying the paper with molybdate–hydrazine; ^{32}P was detected by radioautography. The radioactivity of PP_i was also measured after precipitation as the Mn salt (Kornberg. 1950) after removing nucleotides with charcoal; and that of P_i after precipitation as the molybdate–triethylamine complex (Sugino & Miyoshi, 1964).

The ratio of PP_i to P_i released from ATP increased progressively as the ATP concentration was reduced from 20 to 0·5μM.

At 1μM-ATP the initial rates of formatiou of PP_i and P_i were respectively 147 and 8μμmoles/min.ml. plasma at 37°. The apparent K_m for ATP of the PP_i liberating enzyme was about 2×10^{-7}M. PP_i-liberating ATPases occur in bull semen (Heppel & Hilmoe, 1953) and in cobra venom (Johnson, Kaye, Hems & Krebs, 1953); both these enzymes have some ADPase activity.

When [β-^{32}P]ADP (1μM) was incubated in plasma no PP_i appeared, but P_i was released at an initial rate of 98μμmoles/min./ml. plasma at 37°. This reaction was, therefore, due to an ADPase, rather than to the successive actions of myokinase and ATPase. The K_m of the ADPase for ADP was estimated to be about 1–2μM.

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The Metabolic Fate of ^{35}S-Labelled Heparin and Related Compounds

By A. G. Lloyd,*† G. Embery, F. S. Wusteman and K. S. Dodgson. (*Department of Connective Tissue Research, Institute of Biological and Medical Sciences, Retina Foundation, Boston* 14, *Mass., U.S.A., and M.R.C. Research Group for Studies on Connective Tissues and Lung, Department of Biochemistry, University College, Cardiff*)

The dual labelling of *O*-sulphate and *N*-sulphate (sulphamate) moieties in [^{35}S]heparin prepared by biosynthetic means precludes the use of this material in experiments where specific information is sought concerning factors governing the metabolic integrity of the *N*-sulphate residues of this polymer *in vivo* (cf. Lloyd 1964). On the other hand, evidence has been obtained that re-*N*[^{35}S]-sulphated heparin, prepared by the chemical modification (see Levy & Petracek, 1962) of commercial ox mucous heparin (Evans Medical Co.), is a suitable model for this purpose (Lloyd, Embery, Wusteman, Large & Dodgson, 1964). Preliminary studies (Lloyd *et al.* 1964) revealed that the administration of heparin labelled in this way to experimental animals was followed by the urinary excretion of a significant amount of inorganic $^{35}SO_4^{2-}$ ions.

Extension of this earlier work has shown that when re-*N*[^{35}S]-sulphated heparin (specific radioactivity 0·9mc/mg. atom of S) was given to rats by intraperitoneal injection at a dose level of 0·25mg./animal, 47·6% (±9·4%) of the radioactivity corresponding to the original dose was eliminated in the urine in the first 12hr. after administration. The 12hr. urine increment contained 15·3% (±5·4%) of the injected radioactivity as inorganic $^{35}SO_4^{2-}$ ions. In succeeding 12hr. periods up to 48hr. there was a further excretion of radioactivity corresponding almost exclusively to inorganic $^{35}SO_4^{2-}$. Of the radioactivity excreted during the whole 48hr. period a total of 41·7% appeared in the inorganic sulphate fraction. At higher dose levels (2mg. and 4mg. per animal) the proportion of the dose excreted as inorganic $^{35}SO_4^{2-}$ ions was reduced to 37·1% and 29·5% respectively over the 48hr. period. In the whole experimental series the amount of radioactivity retained by the carcass corresponded to variable values in the range 14·4–27·8% of the injected dose, while the proportion excreted with the faeces was usually small (1·2–5·3%). This pattern of events was not altered appreciably if the ^{35}S-labelled polymer was given intravenously. However, in experiments where the intraperitoneal or intravenous injection of the [^{35}S]heparin was followed immediately by the injection of protamine, urine radioactivity was associated mainly with the inorganic sulphate fraction.

These results contrast sharply with observations made following the intraperitoneal administration of 2[^{35}S]-sulphoamino-2-deoxy-D-glucose (Lloyd, Wusteman, Tudball & Dodgson, 1964) or chitosan *N*[^{35}S]-sulphate (Warner & Coleman, 1958) to rats. In these instances urine radioactivity appeared principally in the ester sulphate fraction and was accompanied by only marginal amounts of inorganic $^{35}SO_4^{2-}$ ions.

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Further Studies on the *N*-Desulphation of [^{35}S]Heparin by Rat Tissues

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The results of studies with re-*N*[^{35}S]-sulphated heparin (Lloyd, Embery, Wusteman & Dodgson, 1966) are consistent with the existence in mam-

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malian tissues of a system capable of separating the *N*-sulphate linkages of this polymer (cf. Danishefsky & Eiber, 1959). An enzyme system having the characteristics of an *N*-sulphatase (sulphamidase) has only been demonstrated previously in cell-free extracts of *Flavobacterium heparinum* cultured on heparin (Korn & Payza, 1956).

In a further series of experiments immobilized rats with bladder and bile duct cannulae were each given 0·8mg. of re-*N*[^{35}S]-sulphated heparin under light ether anaesthesia. The radioactivity of blood samples withdrawn from the peripheral circulation declined to negligible values in the initial 2hr. period after injection. Collection of urine samples at 30min. intervals for a total of 7hr. revealed the excretion of an average of 36·6% of the injected dose in the first 1·5hr., radioactivity being associated solely with the urine ester sulphate fraction. During the remaining 5·5hr. there was a sharp fall in the rate of excretion of radioactivity in the urine yielding a further 22·6% of the dose including a cumulative total of 9·6% as inorganic $^{35}SO_4^{2-}$ ions. As a result of parallel measurements it was found that only 2·2% of the dose, exhibiting the properties of unchanged re-*N*[^{35}S]-sulphated heparin, appeared in the bile during the 7hr. period.

As a corollary to these studies attempts were made to demonstrate the de-*N*-sulphation of the [^{35}S]heparin during the perfusion of blood containing the polymer through isolated preparations of rat liver and kidney. Analysis of samples of blood, bile and 'urine' obtained during the perfusion of these organs failed to reveal the appearance of inorganic $^{35}SO_4^{2-}$ ions.

Treatment of re-*N*[^{35}S]-sulphated heparin with buffered homogenates of rat liver, kidney, lung, aorta, heart, pancreas and skin or suspensions of rat peritoneal mast cells at pH values in the range 4 to 8 did not result in the liberation of inorganic $^{35}SO_4^{2-}$ ions even after periods up 24hr. at 37°. On the other hand, in mixtures containing the polymer plus a 20% (w/v) homogenate of rat spleen in 0·1M-acetate buffer, it was possible to demonstrate the appearance of 16·5–20·1% of the radioactivity as inorganic $^{35}SO_4^{2-}$ ions after 24hr. incubation at 37°. The spleen system was found to be active in the range pH4–5 but not at pH6·0. No de-*N*[^{35}S]-sulphation occurred with spleen homogenates previously heated to 100° for 5min. Essentially similar results were obtained with supernatant preparations obtained after centrifuging 20% (w/v) rat-spleen homogenates at 800*g* for 15min.

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The Self-Irradiation of Carbohydrate *O*-[^{35}S]-Sulphate and *N*[^{35}S]-Sulphate Esters

By A. G. Lloyd,*† E. A. Balazs, G. Embery and F. S. Wusteman. (*Department of Connective Tissue Research, Institute of Biological and Medical Sciences, Retina Foundation, Boston* 14, *Mass., U.S.A., and M.R.C. Research Group for Studies on Connective Tissues and Lung, Department of Biochemistry, University College, Cardiff*)

Carbohydrate *O*[^{35}S]-sulphate and carbohydrate *N*[^{35}S]-sulphate esters of high specific activity prepared by chemical synthesis (Lloyd, 1962*a*; Lloyd, Wusteman, Tudball & Dodgson, 1964; Lloyd, Embery, Wusteman & Dodgson, 1966) have found application as model compounds in the study of the transport and metabolism of biological materials known to contain this type of residue (see for example, Lloyd, 1961, 1962*b*, 1964; Lloyd, Large, James & Dodgson, 1964). During the course of these studies it has become apparent that each of the ^{35}S-labelled esters exhibits an instability which is not characteristic of the corresponding unlabelled analogue.

Observations on factors governing the course of the self-decomposition of labelled sulphate esters have been made using freeze-dried and crystalline preparations of the 6-*O*[^{35}S]-sulphate and di-*O*-[^{35}S]-sulphate esters of D-glucose, D-galactose, *N*-acetyl-D-glucosamine and *N*-acetyl-D-galactosamine, of hyaluronic acid *O*[^{35}S]-sulphate and poly-*O*[^{35}S]-sulphate (Balazs, Hogeberg & Laurent, 1951), and of 2[^{35}S]-sulphoamino-2-deoxy-D-glucose, chitosan *N*[^{35}S]-sulphate, chitosan *O*-sulphate *N*-[^{35}S]-sulphate and re-*N*[^{35}S]-sulphated heparin. Each of these materials, even when maintained *in vacuo* at −20°, showed a progressive liberation of inorganic $^{35}SO_4^{2-}$ attributable to the primary (internal), primary (external) and secondary radiation effects suggested by Phillips (1961). An observed greater lability of the *N*[^{35}S]-sulphates when

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compared with the O[^{35}S]-sulphates suggested that autocatalytic degradation by sulphuric acid liberated in the self-irradiation process also contributed to the degradation of each of these materials (cf. Bayly & Weigel, 1960; Bourne, Hudson & Weigel, 1960).

The self-irradiation processes were virtually eliminated when the esters were kept in dilute, frozen aqueous solution at $-20°$. It must be presumed that when the labelled esters are stored in this way both primary (external) and secondary radiation effects are minimized by a combination of dilution and the limitation of mobility of free radicals (cf. Phillips, 1961).

The finding that carbohydrate O[^{35}S]-sulphate and N[^{35}S]-sulphate esters spontaneously decompose in the solid state giving rise to inorganic $^{35}SO_4^{2-}$ ions stresses the need for care in biological studies based on the use of these materials. This comment also applies to preparations of alkyl O[^{35}S]-sulphates and N[^{35}S]-sulphates, but not to aromatic esters, such as tyrosine O[^{35}S]-sulphate, which were found to be as stable as the corresponding unlabelled esters during extended periods of storage.

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Inhibition of Insulin Action on Muscle by Rat Serum and Liver Extracts

By D. A. B. Young and B. Benson. (*Institut de Biochimie Clinique, Université de Genève, Switzerland*)

Previous work (in the perfused rat heart) has demonstrated the presence of a competitive inhibitor of insulin action on muscle that was under hypothalamic control, and which was postulated to be inactive on adipose tissue (Young, 1965). Later unpublished work indicated that the inhibitor was humoral in origin. To test these findings more directly, rat serum and radioactive glucose, with or without insulin, were injected intraperitoneally into starved weanling rats (Rafaelsen, Lauris & Renold, 1965). The effectiveness of the added insulin as measured by the incorporation of [U-^{14}C]-glucose into diaphragm glycogen was much less when given with normal serum than when given with either serum from hypophysectomized rats or 5% bovine serum albumin. No such difference was observed, however, in the insulin effect on the incorporation of radioactive glucose into lipid of the epididymal fat pad.

The reduction in insulin effectiveness on the diaphragm was found to be due to a substance in the serum, that was associated with the albumin fraction so as to be non-diffusible, and that could be dissociated by boiling either the whole serum or the isolated albumin for 10min. at pH 5·0. When the albumin was prepared under neutral conditions, by precipitation with hexadecylpyridinium bromide (Richter, 1964), the dissociated inhibitor was non-diffusible using Visking tubing 27/32. The substance was stable in acid solution but quickly inactivated in alkaline conditions. It could be partially purified by adsorption from acid solution on to oxycellulose or Amberlite IRC-50, followed by elution with stronger acid. A material that inhibits insulin action on muscle but not on adipose tissue has been found in both rat- and pig-liver extracts and has been partially purified by both the procedures described. It appears to be identical with the serum factor in all the properties so far investigated. In the rat the amount in the whole liver corresponds to 2–3 times the quantity in the total serum.

These findings support the hypothesis (Young, 1965) that there exists a hormone that controls the deposition of fat and its metabolism by restricting in a competitive manner the action of insulin on the uptake of glucose by muscle thereby favouring insulin effect on adipose tissue. The liver may be the source of this endocrine factor, and experiments will be described that attempt to confirm this *in vivo*, and elucidate the role of the pituitary in its control.

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DEMONSTRATION

Microchemical Differentiation Between Optical Isomers of Certain Sugars

By E. G. C. CLARKE and D. J. HUMPHREYS. (*Department of Physiology, Royal Veterinary College, London, N.W. 1*)

D- and L-isomers of sugars give identical osazones, but the DL-form gives an osazone which is quite different in appearance. The shapes of the phenylosazone crystals of various sugars are as follows, those of the optically active form being given first: Altrose, course burrs; feathery rosettes. Arabinose, amorphous or smudge rosettes; rosettes of branching needles. Fucose, amorphous; dense rosettes. Galactose, irregular rosettes; fine feathery crystals. Glucoheptulose, burrs; shell-like rosettes. Glucose, sheaves or fans of needles; bunches of blades or branching needles. Rhamnose, feathery rosettes; irregular blades or needles. Xylose, loose rosettes of long, straight needles; irregular plates or blades. These differences will readily distinguish between the D- and L-isomers on the one hand and the DL-form on the other. The D- and L-isomers may be distinguished from one another by using the method introduced to identify the enantiomorphs of certain narcotic drugs (Clarke, 1958). To a drop of solution of the unknown sugar is added a drop of a solution of one known isomer, say the D-form. The osazone is prepared using the capillary tube method (Clarke, 1956) and examined under the microscope. Should the unknown sugar be the D-isomer, only this form will be present and the crystals seen will be those of the optically active form. Should, however, the unknown be the L-form, both D- and L-forms will be present in the test drop, and the osazone crystals will be those of the racemic form. The method works best if the sugar solutions have a concentration of between 1 and 0·1%. The concentrations of the known and unknown isomers mixed to give the test drop need not be equal, but their ratio should not be greater than 4:1. As the volume required for the test need only be 10 μl., it follows that positive results can be obtained with quantities of sugar as small as 10 μg., which enables the test to be carried out on the amount of sugar that can be eluted from a paper chromatogram.

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **456th Meeting** *of the Society was held in the University of Sheffield on Wednesday and Thursday, 5 and 6 January, 1966, when the following papers were presented:*

COLLOQUIUM ON "CONTROL OF ENZYME PATTERNS IN TISSUES"

The Control of Enzyme Patterns in Animal Tissues

By H. A. KREBS. (*Department of Biochemistry, University of Oxford*)

Decisive progress has been made in recent years in analysing at the molecular level the mechanisms which lie behind the capacity of living organisms to adapt themselves to different physiological situations. The basis of adaptation is in many cases a change in the enzymic equipment of the tissues. There are two main types of mechanisms which bring about such a change. One involves variation in the rate of enzyme synthesis by repression of the formation of some enzymes and induction of the formation of others. The second mechanism involves no variation in the number of enzyme molecules present in the tissue but specific activation and inactivation of given amounts of key enzymes—the allosteric enzymes. The common feature of both mechanisms is the variation of the actual enzyme activities of the tissues. Theoretical aspects of the nature of the two mechanisms and illustrative examples of changes in enzyme patterns brought about by them will be discussed. The examples will be taken from the fields of gluconeogenesis, fatty acid synthesis and nitrogen metabolism.

The Control of Enzyme Patterns in Plant Tissues

By D. D. DAVIES. (*University of East Anglia, Norwich, Norfolk*)

Among the more important biochemical differences between plants and animals is the surplus of reducing potential available to plants. This situation is reflected by the presence of positive feedback systems. These systems will be discussed in relation to the relatively few examples of negative feedback which have been reported in plants.

In addition to the nuclear and mitochondrial DNA of animal cells, plants possess chloroplast DNA and metabolically labile DNA. The relationship between the various types of DNA and the enzymic pattern is not clear. The enzymic pattern is also influenced by at least three hormones—gibberellin, kinetin and indole 3-acetic acid. Current theories place the controlling effect of these hormones at the level of RNA or DNA. Some evidence (Key, 1964) indicates that RNA synthesis and turnover (messenger RNA?) is enhanced by plant hormones, other evidence (Bendana & Galston, 1965) suggests that indoleacetic acid may reduce the rate of RNA degradation.

The application of growth hormones to particular tissues leads to a change in the balance of enzymes. Most published reports indicate a selective increase in particular enzymes, for example tyramine methyl pherase activity in barley is markedly stimulated by kinetin and indoleacetic acid (Steinhart, Mann & Mudd 1964). Examples where this stimulation has commercial significance are the stimulation of latex production in *Hevea* by the application of 2.4-dichlorophenoxyacetic acid and the increase in amylase in barley produced by gibberellic acid. Such evidence will be discussed in relation to reports that kinetin and auxin reduce the relative activities of enzymes of the pentose phosphate pathway.

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The Control of Enzyme Patterns in Yeast

By H. HOLZER. (*Biochemisches Institut der Universität, Freiburg im Breisgau, Germany*)

Mechanisms of control of enzyme patterns in yeast are repression and induction of enzyme synthesis, stabilization (protection against inactivation) and inactivation of enzymes. Regulation of synthesis of NAD-dependent glutamate dehydrogenase (NAD-GluDH) is an example of repression. Many observations suggest that this enzyme is repressed by ammonia or a closely related compound (Holzer & Hierholzer, 1963; Hierholzer & Holzer, 1963). For several reasons to be discussed an induction of the enzyme by glutamate is improbable. A strong indication for repression by ammonia is the appearance of damped oscillations of the rate of

enzyme synthesis after removal of ammonia from the medium in which the yeast cells are growing (Bernhardt, Panten & Holzer, 1964; 1965). Glutamine synthetase is also repressed after addition of ammonia to the growth medium parallel with NAD-GluDH (Kohlhaw, Drägert & Holzer, 1965). Repression of NAD-GluDH is advantageous to the economy of the cells since in the presence of ammonia there is no necessity for the deamination of glutamate. In accordance with this, the NADP-dependent GluDH necessary for glutamate synthesis from ammonia is not repressed. A 'usefulness' for the repression of glutamine synthetase by ammonia is not readily understood. An induction of this ammonia-consuming enzyme by ammonia should be expected. Perhaps there is no (or only a partial) requirement for this enzyme in the presence of ammonia, since all (or at least many) glutamine-dependent amination reactions directly use ammonia. The detour via glutamine is then not necessary. In this context it may be mentioned that glutamine synthetase in *E. coli* is also repressed or inactivated by low concentrations of ammonia (Mecke & Holzer, unpublished results).

A complex form of repression is the 'glucose-effect', which is also called 'catabolite repression' or 'metabolite repression'. An example for this mechanism is the absence of an isoenzyme of malic dehydrogenase (MDH) after growth of yeast with glucose. There are two MDH's in yeast. A cytoplasmic enzyme which is required for gluconeogenesis from acetate via the glyoxylate cycle, and a mitochondrial enzyme which is necessary for the catabolism of glucose via the citric acid cycle. Only the cytoplasmic MDH, not the corresponding enzyme in mitochondria, is absent when the cells are grown on glucose (Witt, Kronau & Holzer, unpublished results).

In intact yeast cells cytoplasmic MDH is rapidly and irreversibly inactivated after addition of glucose to the medium (Witt, Kronau & Holzer, unpublished results). In cell-free extracts this inactivation effect is not observed. *In vivo* it is possible that, in addition to the inactivation of the enzyme, a repression of enzyme synthesis also occurs. However it is also possible that only an inactivation by glucose exists and a repression is only observed as an artifact. This may be true for other cases which have been described as 'repressions'.

For the control of enzyme patterns not only inactivation but also stabilization by metabolites and coenzymes may be of importance. For instance threonine dehydratase is protected by 10^{-4}M-pyridoxal phosphate, 10^{-3}M-valine or 10^{-4}M-isoleucine (Holzer, Boll & Cennamo, 1963; Cennamo, Boll & Holzer, 1964) and glutamine synthetase is stabilized by 10^{-2}M-Mg^{2+} (Kohlhaw *et al.* 1965).

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Changes in Enzyme Patterns in Pathological Conditions

By H. G. Morgan. (*Royal Hospital for Sick Children, Glasgow, C.* 3)

The clinical use of estimations of enzyme activity has increased very greatly during the past decade. (Wilkinson, 1962; Whitby, 1964). Assays are usually on plasma or serum, although enzyme activity is also measured in gut secretions, urine, cerebrospinal and vaginal fluids, internal effusions, tissue biopsies and erythrocytes.

The main indications for assays are: 1. the differential diagnosis of disease—as between hepatitis and bile-duct obstruction. 2. The confirmation of a diagnosis—as in inborn errors of metabolism. 3. To estimate the extent and severity of a disease process—as a gastric carcinoma spread to bone or liver and thus become inoperable. 4. To follow the course of disease and results of specific therapy—as in the treatment of rickets. 5. The detection of minimal degrees of damage when the enzyme assay is the most sensitive index—as in long-term anti-tuberculosis therapy with possible liver damage. 6. Teaching.

Emphasis is now upon changes in the activity of enzymes derived from intracellular activity. The two main sub-groups are alterations in activity that are thought to be the causative factor in the disease process, and, much more frequently, alterations as a result of the disease. The actual levels of activity may be raised or reduced, and, besides technique, several factors are here involved, including release from cells, altered synthesis, catabolism or excretion, and possible changes in inhibitors, activators and cofactors.

The enzymes chosen may also be either specific to a tissue, site or process, or to a varying degree non-specific.

Current usage, with difficulties of clinical interpretation, will be discussed in certain instances. This will include myocardial infarction, (creatine phosphokinase, the transaminases and lactate de-

hydrogenase); liver disease (transaminases, isocitrate dehydrogenase, alkaline phosphatase); pancreas (amylase, leucine aminopeptidase); muscular dystrophy; bone disease; malignancy; and several of the 'inborn errors of metabolism' including Wilson's disease.

Isoenzymes will also be mentioned with their possible use in tissue localization.

It is not so very long since the academic enzymologists 'regarded blood as a contaminant of their tissue preparations and cursed it rather than assayed it' (White, 1958). We are now trying to use the blood to guide us as to the changes in intermediatry cellular metabolism in our patients.

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COMMUNICATIONS

Control of Vitamin B_1 Biosynthesis

By P. C. Newell and R. G. Tucker. (*Microbiology Unit, Department of Biochemistry, University of Oxford*)

Thiamine is formed in micro-organisms by the synthesis and junction of a pyrimidine and a thiazole moiety. Adenosine in high concentration inhibits the biosynthesis of the pyrimidine moiety in *Aerobacter aerogenes* (Moyed, 1964).

We have found that incubation of a growing culture of *Salmonella typhimurium* LT2 with adenosine also causes derepression of the biosynthesis of the pyrimidine. Using this as a tool has enabled us to discover a control mechanism of thiamine biosynthesis in this organism.

When a washed suspension of a tryptophan auxotroph of *Salmonella typhimurium* is incubated in minimal medium lacking tryptophan, little or no thiamine is synthesized. However, if the cells are preincubated in growth medium containing 300μg./ml. adenosine for 2·5hr. then when they are transferred to minimal medium, large amounts of thiamine, 4 to 5 times the normal cell content of 40mμg./mg. dry wt., are synthesized in 1·5hr.

If thiamine is added to the preincubation system then subsequent synthesis of thiamine in non-growing, washed cell suspensions is completely arrested, although thiamine has no action if added after the preincubation. Similar results are obtained if thiamine is replaced by the pyrimidine moiety but not if the thiazole moiety of the vitamin is used.

Thiamine and pyrimidine thus appear to control thiamine formation by repression; the pyrimidine acting either independently of thiamine, or indirectly by being converted into thiamine.

To test this the experiment was repeated on a tryptophan auxotroph also blocked in the formation of the thiazole moiety, which cannot convert added pyrimidine to thiamine. In this system preincubation with adenosine causes the accumulation of large quantities of the pyrimidine, and this is repressible by thiamine. Pyrimidine now has no repressive effect, however, showing that the former effect was indirect.

Similar experiments, performed on another tryptophan auxotroph lacking the ability to join the pyrimidine and thiazole, showed that the thiazole part is not formed in excess when the pyrimidine accumulates. Moreover, thiazole was shown to have its own control, by feedback inhibition rather than repression.

A further control mechanism was also discovered in the enzyme system for joining the pyrimidine and thiazole moieties. Here thiamine again completely represses but a higher concentration is required than for the repression of the pyrimidine pathway, and control of biosynthesis of the pyrimidine and thiazole appears to be the main regulatory mechanism.

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Turnovers of Mitochondrial Components of Normal and Essential Fatty Acid Deficient Rats

By E. Bailey, C. B. Taylor and W. Bartley. (*Medical Research Council Group on Biochemistry and Physiology of Intracellular Organelles, Department of Biochemistry, University of Sheffield*)

In order to test whether mitochondrial lability is altered in essential fatty acid deficiency, turnover rates have been determined for several components of liver mitochondria from deficient and control rats.

Thirty-six newly weaned male rats were fed a synthetic fat-free diet and an equal number of control animals received the same diet supplemented

with 1% corn oil. After 14 weeks each animal received 367 μc [^{32}P]orthophosphate and 27·5 μc [^{35}S]L-methionine by intraperitoneal injection. The rats were killed in groups of six (three deficient and three controls) at intervals up to 5 weeks after injection. Liver mitochondria were fractionated into soluble protein, insoluble protein and lipid by a modification of the method of Fletcher & Sanadi (1961). Total lipid was separated into lecithin, kephalin, cardiolipin and other phospholipids by thin-layer chromatography. The specific activities of ^{32}P in all protein and lipid fractions and the specific activities of ^{35}S in all protein fractions were determined. Turnover rates for the various fractions were calculated from the change of specific activity with time.

There was no significant difference in the rate of change of specific activity of insoluble protein from control and deficient animals, both rates indicating a single component of half-life 9 days. However, for soluble protein in both deficient and control animals there appeared to be two components with half-lives of 4·2 and 15·5 days. The rate of loss of ^{32}P from phospholipids suggested two components with the turnover of both components slower in deficient than control animals. The estimated half-lives of the phospholipids are 3 and 29 days for deficients and 1·6 and 10 days for controls. The relative activities of ^{32}P in the individual phospholipids remained essentially constant during the period in which they were measured (up to 14 days after injection). Thus although total mitochondrial phospholipid turnover was different in the two groups of rats, within a group the turnovers of individual phospholipids were the same.

In deficiency mitochondrial phospholipid turnover was reduced whereas protein turnover was unaffected. The observed differences in the measured half-lives of the various mitochondrial components do not support the view of Fletcher & Sanadi (1961) that mitochondria turnover as an entity.

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Extracellular Release of Lysosomal Enzymes in Response to Sucrose

By Honor B. Fell and J. T. Dingle. (*Strangeways Research Laboratory, Cambridge*)

The injection of sucrose into animals is known to damage the kidneys and liver, producing intense vacuolation of the cells (Janigan, Santamaria & Trump, 1960; Brewer & Heath, 1963). Wattiaux, Wattiaux-de Coninck, Rutgeerts & Tulkens (1965) have shown that in rat liver, sucrose also causes an increase in the size and fragility of the lysosomes.

In the present study, the effect of sucrose on embryonic (chick) cartilage and bone grown in organ culture has been investigated. Sucrose was readily taken up by the explants (38 μg. sucrose/μg. DNA phosphorus) but was not appreciably metabolized. It greatly increased both the release and total synthesis of lysosomal acid protease and acid phosphatase; for example when 7-day (cartilaginous) limb-bone rudiments were cultivated for 8 days in medium containing 0·08 M-sucrose, they released an acid proteolytic activity of 325 μg. tyrosine/hr./rudiment as compared with 46 μg. of tyrosine/hr./control rudiment without sucrose. The total enzyme synthesized (activity of rudiment + activity of medium) was 438 μg. tyrosine/hr./bone grown in the presence of sucrose, and that of the controls 118 μg. tyrosine/hr./rudiment. Histological examination showed that the cytoplasm of the connective tissue cells surrounding the cartilage was distended with small vacuoles which probably contained sucrose, though this has not yet been demonstrated; the cells appeared healthy and many mitoses were present. In the cartilage itself, this abnormal vacuolation appeared only near the articular surface and here the matrix was breaking down.

Sucrose produced similar cytological and biochemical changes in bone from 12–14 day embryos, but in such explants even the osteocytes were highly vacuolated and the well calcified matrix was undergoing a curious lysis.

When embryonic cartilage and bone are cultivated in medium containing complement-sufficient antiserum, the matrix breaks down, and the release of lysosomal enzymes is increased (Fell, Coombs & Dingle, unpublished). The addition of sucrose to medium containing a weak antiserum greatly enhanced both the dissolution of the intercellular material and the synthesis and release of acid proteolytic activity in explants of 7-day limb-bone rudiments; in these explants, unlike those with normal serum, cytoplasmic vacuolation extended far into the cartilage.

In preliminary experiments mannitol produced changes similar to those of sucrose, but glucose in the same concentration did not. The results suggest that the accumulation of undigested material in cells stimulates the release as well as the synthesis of lysosomal enzymes, a phenomenon that may prove important in pathology.

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Changes in the Carbohydrate Content of Rat-Heart Slices

By W. Bartley and B. Dean. (*Department of Biochemistry, University of Sheffield*)

Changes in the carbohydrate content of rat-heart slices have been studied under various incubation conditions. Rats were anaesthetized with ether and nembutal, the heart was excised whilst still beating and then chilled in saline at 0°. Heart slices were cut by hand and the slices chopped transversely with a McIlwain chopper. The heart ribbons were then incubated in a suitable saline and at the end of incubation the vessel contents were boiled for 15min. to stop enzymic actions and to solubilize the carbohydrates. The mean value before incubation on average of the sum of 'glycogen' and glucose (measured as glucose by successive treatment with 'Diazyme' and glucose oxidase, see Pazur & Ando, 1959, 1960, Krebs, Bennett, de Gasquet, Gascoyne & Yoshida, 1963) in the heart was 20·7 μmoles/g. dry wt. and of this 3·6 μmoles/g. dry wt. was free glucose. Isolation of the tissue glycogen by the method of Walaas & Walaas (1950) and subsequent treatment with 'Diazyme' and glucose oxidase gave a mean value of 12·3 μmoles of glycogen measured as glucose/g. dry wt., whereas 16·5 μmoles/g. dry wt. would be expected from the difference between total and free glucose. After incubation for 1hr. in Krebs–Henseleit saline at 37°, the total glucose increased on average by 27·7 μmoles/g. dry wt., the free glucose remained virtually unchanged and the glycogen decreased by 11·2 μmoles/g. dry wt. During incubation of heart therefore, some glucose-containing material is produced which, after treatment with 'Diazyme', gives free glucose. The amounts of copper-reducing material (Nelson, 1944) also increased on incubation by 19·5 μmoles/g. dry wt. (calculated with glucose as a standard). Treatment with 'Diazyme' before estimation of the copper reducing equivalents raised the amount to approximately the value of total glucose measured by glucose oxidase. The production of carbohydrate by incubated heart was not influenced by adding known precursors of glucose, nor by anaerobic conditions, but the amount formed was decreased 75% by the presence of adrenaline (2×10^{-4}M) and 80% by 25mM-phosphate. Homogenates of heart behave in a similar way to the slices, but the changes are much more rapid. Estimation of the amounts of hexose phosphate in the heart gave values which at most could account for only 10% of the glucose-containing substance that was formed on incubation.

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Nicotinamide–Adenine Nucleotides during the Life Cycle of the Blowfly, *Lucilia cuprina*

By L. M. Birt. (*Department of Biochemistry, University of Sheffield*)

Variations in the amounts and distribution of nicotinamide nucleotides during the life cycle of *Lucilia* have been estimated by an adaptation of the spectrophotometric assay of Villee (1962). Both NAD and NADP declined during the transformation from prepupae to midpupae (from 330 and 31 mμmoles/g. fresh wt. to 190 and 20 mμmoles/g. fresh wt. respectively) and both reappeared rapidly during adult development, the amount of NAD increasing three-fold and of NADP two-fold in the mature fly. The ratio NADH/NAD^+ was relatively constant (0·14–0·16) except during the latter half of pupal life (0·09); that of NADPH/$NADP^+$ was lower (0·61) during larvae life and in mated adult flies than at all other stages (0·85–0·95). During adult development, the rate of increase of NAD was greatest in the thorax, which finally contained about 65% of the total NAD; the level of NADP was greatest in the abdomen. NAD in homogenates of tissues sampled 2 days before eclosion was destroyed relatively rapidly even in the presence of nicotinamide (50% loss in 15min. at 25°) but with tissue from adults the loss was much less (10%); NADP was stable in both types of preparation. In the thorax of the adult, about 40% of the NAD and 50% of the NADP, was particulate. The incorporation of NAD into flight muscle mitochondria began about 1 day before eclosion and proceeded rapidly for 2days. Initially, most of the particulate NAD (77%) was present in particles 0·2–1 μ in diameter and of relatively high specific content (7·8mμmoles/mg. protein); during development, there was a gradual shift of NAD to larger particles (1–10 μ) containing about 4mμmoles/mg. protein (cf. Birt, 1961). A similar, but less marked change, occurred with NADP, the final concentration in the larger particles being about 0·2 mμmoles/mg. protein. It seems probable that this pattern reflects the incorporation of nucleotides into the fully developed sarcosomes through a precursor stage (the smaller particles).

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Changes in the Activity of Respiratory Enzymes during the Development of the Blow-Fly, *Lucilia cuprina*

By R. W. Lennie and L. M. Birt. (*Department of Biochemistry, University of Sheffield*)

The changes in amounts of several mitochondrial enzymes have been studied during the development and growth of *Lucilia*. Homogenates of whole insects or thoraces were fractionated into different sized particles by centrifuging (Smith, 1961). A spectrophotometric method was used for dehydrogenase assay (Lee & Lardy, 1965) which gave rates of electron transfer (calculated from formazan production and expressed in units of μl. O_2/mg. protein/hr.) comparable to values reported for isolated mitochondria.

Succinate, α-glycerophosphate (FAD-dependent), isocitrate (NAD- and NADP- dependent) and malate (NAD-dependent) dehydrogenases and cytochrome *c* oxidase were assayed in the particulate and soluble fractions, from a half-day before emergence to maturation of the fly (7–8 days). The structurally-bound enzymes (cytochrome *c* oxidase, α-glycerophosphate and succinate dehydrogenases) developed synchronously, increasing in activity between two- and three-fold. Most of the initial increase occurred in a small particle fraction (0·2μ–1μ) of high specific activity which was maximal at emergence (400 and 120 units with α-glycerophosphate and succinate respectively), but which declined in the mature fly (200 and 50 units respectively). The activities in the soluble fractions never exceeded 15% of the total activity.

Similar patterns of change in activities and specific activities were recorded for the NAD-dependent isocitrate and malate dehydrogenases, the activities increasing by 5·5-fold and 40% respectively. However, a larger proportion of total activity was detected in the soluble fraction (about 20% and 30% respectively). In sharp contrast the activity of the NADP-dependent isocitrate dehydrogenase did not alter and was present almost exclusively in the soluble fraction.

Thus, a rapid and synchronous synthesis of mitochondrial enzymes occurred during the period of emergence, and a small particle fraction appeared to act as a precursor for the development of the larger particles.

The results will be discussed in relation to the morphogenesis of the mitochondria of flight muscles, and the physiological significance of their morphology.

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Changes in the Lipid Content during Metamorphosis of the Blowfly, *Lucilia cuprina*

By M. A. D'Costa and L. M. Birt. (*Department of Biochemistry, The University of Sheffield*)

Changes in the lipids of *Lucilia* during its life cycle have been estimated. The amount of phospholipid (as μg. lipid phosphorus per organism) increased from a prepupal value of 13 to 19 (1-day old pupae), fell to 12 in midpupal life and rose to 19 at emergence. The phospholipid was mainly phosphatidylethanolamine and phosphatidylcholine (cf. Bridges & Cox, 1962; Crone & Bridges, 1963; Gilby, 1964) with a fatty acid composition of about 50% saturated acids (mainly C_{16}, C_{18} and compounds less than C_{14}) and 50% unsaturated ($C_{16:1}$ and $C_{18:1}$).

Tri- and di-glycerides present in prepupae (approx. 10 and 0·35 μmole of ester per organism respectively) decreased immediately after pupation by about 20% then rose to about the original value at which they were maintained during midpupal life. Just before emergence they declined by approx. 90% each adult fly containing only 1 μmole neutral lipid. The glycerides contained mainly saturated acids (65–90% of the total) chiefly C_{14}, C_{16} and C_{18}. Both saturated and unsaturated fatty acids of the glycerides declined initially at the same rate, but the subsequent changes involved only the saturated acids.

Over the period of emergence the glycerides in both the abdomen and thorax declined from 3·9 to 1·3 and from 2·7 to 2·3 μmoles of ester per individual respectively.

The total amount of free fatty acids at all stages of the life cycle was very low (about 25 mμmoles per insect).

The results suggest that the consumption of glycerides serves as the main energy source for synthetic activities occurring immediately prior to emergence. Amino acids and carbohydrates appear to be more important in the earlier stages of development.

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The Synergistic Decarboxylation of Glyoxylate and 2-Oxoglutarate by an Enzyme from Mammalian Liver

By P. R. STEWART and J. R. QUAYLE*. (*Department of Biochemistry, University of Sheffield*)

Glyoxylate is metabolised by a number of pathways in mammalian tissues. It may be oxidized to oxalate or reduced to glycollate (Runyan & Gershoff, 1965). There are enzymic and non-enzymic mechanisms for its amination to glycine, and for its condensation with keto acids. (Nakada, 1964; Kuratomi & Fukunaga, 1960; Ruffo, Adinolfi, Budillon & Capobianco, 1962). Two reports have been given of the decarboxylation of glyoxylate in mammalian systems. Nakada & Sund (1958), using rat-liver homogenates, described the decarboxylation of glyoxylate in the presence of L-glutamate; *N*-formyl glutamate was isolated as a product. Crawhall & Watts (1962), on the other hand, observed a similar decarboxylation by mammalian mitochondria, but were unable to detect *N*-formyl glutamate as an intermediate or product of the reaction. In addition, these latter workers noted than 2-oxoglutarate could replace L-glutamate.

We have partially purified an enzyme from pig-liver mitochondria which catalyses a synergistic decarboxylation of glyoxylate and 2-oxoglutarate. The enzyme has been purified more than 100-fold by fractional precipitation with ammonium sulphate and chromatography on columns of Sephadex G-200, calcium phosphate/cellulose, and DEAE-cellulose. The purified system is unstable but its lability is decreased in the presence of sucrose, mercaptoethanol, and magnesium chloride. The enzyme is inhibited by *N*-ethylmaleimide and EDTA but not by avidin. Activity is not affected by the addition of substrate amounts of NAD, NADP, ATP, CoA or thiamine pyrophosphate. The substrate analogues pyruvate, glycollate, glycolaldehyde, malonate, glycine and L-glutamate will not replace glyoxylate or 2-oxoglutarate as substrates for decarboxylation. Nor do these substances behave as inhibitors in the reaction.

When [$^{14}C_1$]glyoxylate or 2-oxo[$^{14}C_1$]glutarate are used as substrates, radioactive carbon dioxide is recovered. [$^{14}C_2$]Glyoxylate or 2-oxo[$^{14}C_5$]glutarate do not yield labelled carbon dioxide, but are recovered in an end-product not yet completely characterized.

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The Non-Protein Nitrogenous Constituents of the Maternal Blood and Foetal Body Fluids in the Sheep

By A. ST. G. HUGGETT and J. S. SLATER. (*Animal Diseases Association, Moredun Institute, Gilmerton, Edinburgh*)

Non-protein nitrogen (NPN) and free neutral and acidic amino acids have been determined in the maternal blood plasma, foetal blood plasma, allantoic and amniotic fluids of six sheep at different stages of pregnancy from the 79th day of gestation, and in four lambs aged between 1 and 34 days.

Ion-exchange chromatography of protein-free extracts confirmed that, as in the guinea-pig and rabbit (Christensen & Streicher 1948) and in the human (Ghadimi & Pecora, 1964), amino acid concentrations were higher in foetal than in maternal plasma. The foetal/maternal ratio for NPN ranged from 1·34 to 1·80; for individual amino acids the ratio was occasionally less than 1, but generally greater than 1, ranging to 3 in some cases. The effect was strongly in evidence at the 79th day of gestation. These ratios derived from low maternal and high foetal levels, relative to the normal adult range. Although the graphs of foetal age against NPN were very similar for foetal and maternal plasma, the same did not always apply to time-plots of individual amino acid concentrations.

Non-protein nitrogen and amino acids of the 1-day old lamb were more concentrated than in the full-term foetus, but thereafter decreased towards the adult level at 34 days of age.

Seven unidentified ninhydrin-reactive components of the foetal plasma were not detectable in maternal plasma, but occurred in allantoic fluid in considerably higher concentrations than in the foetal plasma. The allantoic NPN was 3 to 8 times that of the foetal plasma, with highest values at 100 to 120 days, falling to a value close to that of the plasma at term. In contrast to the maternal and foetal plasmas the allantoic time-plots for individual amino acids were similar in shape to that of the total NPN.

The NPN concentration of amniotic fluid rose slowly during pregnancy. The main nitrogenous component was urea, with traces only of amino

acids. In the sheep examined at about full term, the amniotic fluid contained greatly increased quantities of all amino acids. This sheep was, however, found later to be approximately a week past full-term. Since the foetal age of the previous sheep was 121 days it is not known how much significance can be attached to these quantities. In considering the significance of concentrations we can state that, so far as plasma is concerned, they are the resultants of inflow and outflow.

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Glucose Catabolism in the Cotyledons of Germinating Pea Seeds

By J. L. Wray and A. P. Brown. (*Biochemistry Department, University of Birmingham*)

Developmental changes have been examined in the enzymic activities of hexokinase, phosphofructokinase, aldolase and glucose 6-phosphate/6-phosphogluconate dehydrogenases in the pea cotyledon during germination. As germination proceeded, the enzymes examined, with the exception of aldolase, showed a peak of activity occurring at different times, followed by a decline. The maximum activity of phosphofructokinase and glucose 6-phosphate/6-phosphogluconate dehydrogenases occurred at 3 and 4 days respectively, whereas that of hexokinase occurred at about 6 days. The aldolase activity decreased without showing any peak activity. The activities of aldolase, phosphofructokinase and glucose 6-phosphate/6-phosphogluconate dehydrogenases were all considerably higher than that of hexokinase at all stages of germination, so that entry of glucose into both glycolytic and pentose phosphate pathways is probably controlled by the activity of the common enzyme, hexokinase.

The ATP content of the cotyledons decreased during germination until at 8 days the value (0·15 μmoles ATP/g. fresh wt.) was about one-third that at 1 day. The values were such that the rate of both the hexokinase and phosphofructokinase reactions could be expected to be limited by ATP concentration. Since the K_m (ATP) for hexokinase (wheat germ) (Saltman, 1953) is 0·87mM and for phosphofructokinase (pea) (Axelrod, Saltman, Bandurski & Baker, 1952) is 2·4mM, the effect of ATP limitation would be greater at the phosphofructokinase level, and might provide a control mechanism for regulating glucose metabolism via the glycolytic pathway. The low ATP level reinforces the suggestion that entry of glucose into the two pathways is controlled at the hexokinase level.

In a variety of immature tissues glucose is catabolized to a large extent via the glycolytic pathway, but as the tissue ages participation of the pentose phosphate pathway becomes increasingly pronounced (Beevers & Gibbs, 1954; Gibbs & Beevers, 1955). Preliminary investigations in this laboratory using specifically labelled radioactive glucoses have shown that, as germination proceeds, glucose catabolism via the pentose phosphate pathway becomes increasingly important. As the development of this pathway is not controlled by the enzymic content of the tissue alone, it appears that cofactor concentration must also be considered as a factor regulating glucose catabolism.

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Urinary β-Galactosidase and Kidney Damage

By N. Dance, R. G. Price and D. Robinson. (*Department of Nutrition, Queen Elizabeth College, University of London, W.8.*)

The excretion of β-galactosidase in the urine of normal adult rats has been previously reported (Furth & Robinson, 1965); the daily output is sufficient to hydrolyse 4·5 μmoles of 4-methylumbelliferyl β-D-galactoside/hr. under optimum assay conditions. *N*-Acetyl-β-glucosaminidase and β-glucosidase can also be detected. Although the elution patterns from DEAE-cellulose columns showed a close resemblance between the urinary enzyme and that from kidney, gel electrophoresis at pH 7·0 gives different patterns for the two sources.

The urine was concentrated about 40-fold by dialysis under reduced pressure against gel buffer (0·04M-phosphate). Both urine and kidney had slow moving components, but in addition kidney homogenates had a distinctive sharp band of activity which migrated rapidly towards the anode. Both β-galactosidase and β-glucosidase could be detected in this band by flooding the gels with a solution of the appropriate 4-methylumbelliferyl-glycoside. Fluorescent bands were readily visible under an ultraviolet lamp after 5 to 10min. at 37°. The presence of glucono(1→5)-lactone in the developing solution inhibited the detection of both β-glucosidase and β-galactosidase in the fast-moving

band, but had no effect on the slow-moving β-galactosidase components. The bands may thus differ in their substrate specificities.

When kidneys were damaged by uranium nitrate (50mg./kg. intraperitoneally) there was a threefold rise in urinary glycosidase levels and this was accompanied by proteinuria and diuresis. The effect of *p*-nitrophenyl arsonic acid at the same dose level was even more pronounced, with enzyme levels rising to ten times the normal value.

When these urines were examined by gel electrophoresis they showed the same band of fast mobility which had been seen with extracts of normal kidney.

This new urinary component appeared within 24hr. and gradually diminished over 3 days after dosage, by which time the enzyme levels had returned to normal. A similar but much less pronounced effect was obtained by the injection of aspirin (200mg./kg.).

Uranium nitrate causes necrosis of the proximal convoluted tubules (Tapp, Carroll & Kojacs, 1965), and these stain strongly for β-galactosidase (Rutenburg, Rutenburg, Monis, Teague & Seligman, 1958). Aspirin on the other hand is said to remove degenerate cells from the tubules by the irritant action of the drug (Scott, Denman & Dorling, 1963).

Experiments are in progress to determine the nature of this fast band. Chromatography of kidney extracts on Sephadex G-75 in 0·01M-phosphate buffer, pH7·6, containing 0·4M-NaCl, gave an asymmetric peak. Gel electrophoresis of subfractions from this peak showed the slow-moving component was eluted before the fast band. Gel filtration on Sephadex G-200 (Andrews, 1965) suggests a molecular weight of approximately 100000 for the main slow-moving components, while the fast band is about half this size.

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Transfer of Iodide by the Small Intestine of the Rat *in vitro*

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The central region of rat small intestine transfers iodide from the serosal to the mucosal side *in vivo* (Pastan, 1957) and *in vitro* (Acland & Illman, 1959). Iodide transfer by the rat jejunum and ileum was further studied by the everted sac technique (Wilson & Wiseman, 1954; Wiseman, 1961). The small intestine was divided into five portions of equal length. The corresponding everted sacs were numbered from the duodenal end. All media contained initially glucose (0·017M), $^{127}I^-$ (0·1m-equiv./l.) and $^{131}I^-$ (0·1–0·2μc/ml.). Radioactivity measurements were used to determine the final iodide concentrations after 1hr. at 37° (shaking rate 84 oscillations/min.).

In seven experiments, no important difference was found between the rates of net mucosal iodide transfer in Krebs' bicarbonate and phosphate buffers (Krebs & Henseleit, 1932; Krebs, 1933). Ranges (μ-equiv./hr.) in bicarbonate buffer were: *Sac* 1, 0·038 to 0·107; *Sac* 2, 0·214 to 0·246; *Sac* 3, 0·231 to 0·300; *Sac* 4, 0·056 to 0·206; *Sac* 5, −0·052 to 0·111. Ranges (μ-equiv./hr.) in phosphate buffer were: *Sac* 1, −0·044 to 0·109; *Sac* 2, 0·077 to 0·283; *Sac* 3, 0·245 to 0·322; *Sac* 4, 0·065 to 0·165; *Sac* 5, −0·037 to 0·122.

In nine experiments, net serosal iodide transfer was observed in a chloride-free bicarbonate buffer (0·724% w/v Na_2SO_4—100 ml.; 0·972% w/v K_2SO_4—4ml.; 0·955% w/v $MgSO_4.7H_2O$—4ml.; 2·11% w/v KH_2PO_4—1ml.; CO_2-saturated 1·3% w/v $NaHCO_3$—21ml.; Ca gluconate—0·1425g.; mannitol—1·456g.) and in a chloride-free phosphate buffer (0·724% w/v Na_2SO_4—100ml.; 0·972% w/v K_2SO_4—4ml.; 0·955% w/v $MgSO_4,7H_2O$—4ml.; phosphate buffer containing 40ml. of 0·25M-Na_2HPO_4 and 2ml. of 1N-H_2SO_4 in 100ml.—21ml.; Ca gluconate—0·1425g.; mannitol—1·492g.). In *Sac* 5, the transfer rate was greater in the phosphate than in the bicarbonate buffer. Ranges (μ-equiv./hr.) in chloride-free bicarbonate buffer were: *Sac* 1, 0·065 to 0·336; *Sac* 2, 0·016 to 0·201; *Sac* 3, 0·059 to 0·229; *Sac* 4, 0·226 to 0·438; *Sac* 5, 0·188 to 0·441. Ranges (μ-equiv./hr.) in chloride-free phosphate buffer were: *Sac* 1, 0·160 to 0·243; *Sac* 2, −0·001 to 0·176; *Sac* 3, 0·071 to 0·151; *Sac* 4, 0·217 to 0·458; *Sac* 5, 0·400 to 0·724.

The significance of these results will be discussed.

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The Action of Semicarbazide on the Aggregation of the Tropocollagen Macromolecule

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Acid-soluble collagen solutions, pH 4·2, prepared according to the method of Wood & Keech (1960), were treated with semicarbazide hydrochloride for 30 min. at temperatures from 25° to 40°. The final pH of the incubation mixture was 2·7. Controls, substituting sodium chloride for semicarbazide, were similarly treated at pH 4.2. Test and control solutions were dialysed exhaustively against acetate buffer, pH 4·2 and containing sodium chloride, and studied as follows:

Ultraviolet difference spectra between test and control solutions were recorded, and the height of the positive peak observed at 220 mμ was measured.

In vitro fibril formation was studied using the method of Wood & Keech (1960) and the final extinction value, denoting complete precipitation and extent of lateral aggregation was recorded for the tests (E_{∞_t}) and controls (E_{∞_c}). E_{∞_t} fell considerably in solutions treated above 32·1°.

A plot of ($E_{\infty_t} - E_{\infty_c}$) against the corresponding treatment temperature resulted in a curve which was identical to that produced when the 220 mμ peak height was plotted against temperature. This indicated a correlation between lateral aggregation and conformational changes.

Study of the behaviour of collagen solutions adjusted to pH 2·7 with hydrochloric acid showed that the effect of semicarbazide could not be wholly attributed to a pH effect.

Experiments were also performed using semicarbazide, buffered at various pH values from 4·2 to 8·9. Between 4·2 and 4·5, E_{∞_t} exceeded E_{∞_c}, whereas above pH 4·5 the reverse was true. In all cases, precipitation was more extensive than at pH 2·7.

Acid-soluble collagen solutions were also treated at 37°, pH 2·7, with ^{14}C-labelled semicarbazide hydrochloride. After the removal of the excess semicarbazide by dialysis or gel filtration, approximately 6 moles of semicarbazide were bound per mole of tropocollagen.

Semicarbazide appears to affect the degree of lateral aggregation which is dependent on pH and temperature in a complex manner. The correlation of this degree of lateral aggregation with the conformational changes will be discussed. A comparison of the results with those of Wood (1963) will be made.

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β-Xylosidases of Animal and Other Sources in Relation to the Degradation of Chondroitin sulphate-Peptide Complexes

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The recognition of xylosyl-serine linkages in various chondroitin sulphate-protein complexes (Rodén, Gregory & Laurent, 1963; Gregory, Laurent & Rodén, 1964; Kent & Stevenson, 1963, 1965; Anderson, Hoffman & Meyer, 1965) suggests that β-xylosidases, may be present in animal tissues and may play a significant part in the enzymic breakdown of these connective tissue constituents.

Hitherto, β-xylosidase activity has been reported as accompanying β-glucosidase activity in bacterial and plant preparations, without decisive evidence whether two types of enzyme exist or whether a single enzyme of broad specificity is involved. It has now become possible to examine this question and to detect β-xylosidase activity readily by the use of two model substrates *viz.* *o*-nitrophenyl-β-D-xyloside and *p*-nitrophenyl-β-D-xyloside. These compounds were synthesized in the usual way by reaction of tri-*O*-acetyl-α-D-xylopyranosyl bromide (Weygand, 1962) with the respective nitrophenol in alkaline acetone (Synder & Link, 1952). The *o*-nitrophenyl tri-*O*-acetylglycoside had m.p. 115° $[\alpha]_D^{20} - 40°$, (*c* 0·7 in ethanol) and had a proton magnetic resonance spectrum ($CDCl_3$, 60 m./c.) showing a doublet at τ 6·5, with a splitting of 0·7 p.p.m. consistent with a β-glycoside structure. Catalytic deacetylation gave *o*-nitrophenyl-β-D-xyloside (m.p. 171–172°; $[\alpha]_D^{20} - 86·9°$, *c* 0·5 in H_2O). The corresponding *p*-nitrophenyl tri-*O*-acetyl-β-D-xyloside had m.p. 149° ($[\alpha]_D^{25} - 43·1°$, *c* 0·4 in ethanol) and *p*-nitrophenyl-β-D-xyloside m.p. 160° $[\alpha]_D^{25} - 53·1°$, *c* 0·4 in H_2O.

Using the *o*-nitrophenylxyloside, almond emulsin (Koch–Light Laboratories, 620 units/mg.) was found by gel-filtration on Sephadex G-100 to contain two xylosidase-active protein constituents. In these, the ratios of β-glucosidase to β-xylosidase were 2.6:1 and 8:1 respectively (measured in sodium acetate buffer at pH 5·2 and at 30°). The powerful β-glucosidase from the right collateral gland of *Periplaneta americana* (Brunet & Kent, 1955) was devoid of xylosidase activity.

Work in collaboration with Dr A. Barrett and Dr J. T. Dingle of the Strangeways Laboratory Cambridge, has demonstrated that particulate preparations of rat liver, sedimented at 10000 ***g*** for 15 min., possess β-xylosidase activity, with an optimum at pH 5·0. Sucrose-layering techniques gave a particulate preparation with increased specific activity, which was paralleled by increase

in specific acid phosphatase activity. The particulate preparation was inactive unless lysed with water or detergent. This evidence suggests that the β-xylosidase of rat liver is found within lysosome particles. Only very low levels of activity could be found in preparations from chick-embryo cartilage.

Preliminary studies, indicate that the DNP-chondropeptide obtained from bovine tracheal cartilage by papain digestion (Kent & Stevenson, 1963, 1965) is hydrolysed by β-xylosidase from emulsin at the point of the xylose-serine linkage.

Xylose-containing oligosaccharides (detected on the basis of a modified anthrone reaction) and DNP-peptides resulting from the cleavage may be separated chromatographically on talc (Sanger, 1949).

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